CHEMICAL OXIDATION OF AQUATIC ANTIBIOTIC MICROCONTAMINANTS BY FREE AND COMBINED CHLORINE

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CHEMICAL OXIDATION OF AQUATIC ANTIBIOTIC MICROCONTAMINANTS BY FREE AND COMBINED CHLORINE

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DEDICATION

With utmost respect and love, I dedicate this work to my Grandparents Reese, who have stood steadfast by my side for nearly seven years as I've labored through the gauntlet that is the Georgia Institute of Technology. Their unending support, seasoned wisdom, and Brunswick stew have seen me through some very difficult times. Never once has their faith in me wavered.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMI – 3-amino-5-methylisoxazole

AOP – Advanced oxidation process

APCI – Atmospheric pressure chemical ionization

APMS – 4-aminophenyl methylsulfone

CC – Combined chlorine

CF – Ciprofloxacin

D.I. – Deionized

DAD – Diode-array detector

DAMP – 2,4-diamino-5-methylpyrimidine

DCMP – 2,4-dichloro-5-methylpyrimidine

DHFR – dihydrofolate reductase inhibitors

DMI – 3,5-dimethylisoxazole

DPD – N,N-diethyl-*p*-phenylenediamine

EF – Enrofloxacin

ESI – Electrospray ionization

FAC – Free available chlorine

FAS – Ferrous ammonium sulfate

FLD – Fluorescence detection

FLU – Flumequine

FQ – Fluoroquinolone

GC/MS – Gas chromatograph/mass spectrometer

HLB – Hydrophilic-lipophilic balance

HPLC – High-performance liquid chromatograph

k_{app}' – Apparent pseudo-first order kinetic rate constant

k_{app}'' – Apparent second order kinetic rate constant

LC/MS – Liquid chromatograph/mass spectrometer

LF – Lomefloxacin

MMIB - 4-methyl-N-(5-methyl-isoxazol-3-yl)-benzenesulfonamide

NMR – Nuclear magnetic resonance

NPOC – Non-purgeable organic carbon

OF – Ofloxacin

PCA – Principal component analysis

PhAC – Pharmaceutically active compound

pK_a (or K_a)– Acid ionization, or dissociation constant

SMX – Sulfamethoxazole

SPE – Solid phase extraction

$t_{1/2}$ – Half-life

THAM – Tris-hydroxymethyl aminomethane

TMP – Trimethoprim

TMT – 3,4,5-trimethoxytoluene

TOC – Total organic carbon

UV/Vis – Ultraviolet/Visible light

WRF – Wastewater reclamation facility

WTP – Water treatment plant

a_0 – Substrate species 0 dissociation coefficient

a_0' – HOCl dissociation coefficient

a_1 – Substrate species 1 dissociation coefficient

a_1' – OCl^- dissociation coefficient

a_2 – Substrate species 2 dissociation coefficient

SUMMARY

Chapter I. This study presents reaction kinetics, mechanisms, and proposed degradation pathways of the reactions between sodium hypochlorite (free available chlorine – FAC) or chloramines (combined chlorine – CC) and four fluoroquinolone (FQ) antibiotics (Table I-1) – ciprofloxacin (CF), lomefloxacin (LF), ofloxacin (OF), and enrofloxacin (EF). Reactions were initiated by addition of sodium hypochlorite or pre-formed chloramines (in 10:1 or 11:1 oxidant to antibiotic molar ratio) to each antibiotic compound in acetate-, phosphate-, or borate-buffered solutions at pH values ranging from 4 to 9. Reactions were monitored by quenching 1-mL samples of each reaction solution at appropriate time intervals and analyzing by HPLC with fluorescence detection. Sodium thiosulfate was used to consume residual oxidant in kinetic experiments involving both free available chlorine and combined chlorine. Pseudo-first-order kinetics was observed for oxidation of the antibiotics OF and EF by chlorine, while pseudo-first-order kinetics was also observed for decay of primary intermediates formed via reaction of CF and LF with FAC. Second order kinetic rate constants for reactions involving OF and EF were calculated from observed pseudo-first order constants, on the assumption that concentration of oxidant remained essentially constant throughout the monitoring periods of each kinetic experiment. CF was directly oxidized by FAC at a reaction rate too fast (k_{app}'' >> 5.4 x 10^3 $M^{-1}s^{-1}$ at pH 7) to quantify by analytical techniques available during the course of this study. OF and EF were directly oxidized at significantly lower rates (6.3 x 10^3 $M^{-1}s^{-1}$ and 4.7 x 10^2 $M^{-1}s^{-1}$, respectively, at pH 7). Solution pH exhibited a marked influence on reaction rates for OF

and EF. Consideration of predominant antibiotic species and corresponding reaction rates at specific pH values allowed a preliminary determination of reactive sites within the antibiotics. Additionally, the decay rate of primary N-chlorinated intermediates of CF and LF was determined on the basis of their reconversion to CF or LF upon addition of $Na_2S_2O_3$ to reaction solutions. A structurally-related compound – flumequine was also examined in order to verify the proposed site(s) of reaction. LC/MS was utilized to identify reaction products and to assess product evolution during each reaction time course. Product characterization and temporal distribution provided further insight into reaction pathways and mechanisms. In addition, oxidation of CF in real environmental matrices was used to assess applicability of findings based on the above laboratory studies. The results of this study indicate that representative members of the fluoroquinolone antibiotic class are substantially degraded under conditions simulating chlorination of water supplies during disinfection processes. Maximum reaction rates are observed for each of the compounds under conditions likely to be encountered in water treatment facilities (i.e., pH values between 6 and 8). CF was substantially degraded in real wastewater and drinking water samples under conditions applied during disinfection at a municipal wastewater reclamation facility and drinking water treatment facility, respectively.. Kinetic data (for surrogate compounds, as well as CF) and structural characterization of oxidation products have been utilized here to develop general degradation pathways for reaction of fluoroquinolone antibiotics with chlorine-based oxidants.

Chapter II. This study presents reaction kinetics, mechanisms, and proposed degradation pathways of the reactions between sodium hypochlorite or chloramines and the environmentally-

significant (i.e., detected frequently in municipal wastewater effluents and natural surface waters) antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP). Reactions were initiated by addition of sodium hypochlorite or pre-formed chloramines (in 10:1 oxidant to antibiotic molar ratio) to the respective antibiotic compounds in acetate-, phosphate-, or borate-buffered solutions at pH values ranging from 3 to 9. Reactions were monitored by quenching 1-mL samples of each reaction solution at appropriate time intervals and analyzing by HPLC with UV detection. Samples obtained from kinetic experiments involving FAC were prepared for analyses using "soft" quenching, incorporating a combination of NH_4Cl, tris-hydroxymethyl aminomethane, and CH_3COOH. Reactions of SMX and TMP with chlorine yield N-chloro intermediates that can be easily reduced and converted back to the parent compound by strong reductants such as sodium thiosulfate. Experiments showed that NH_4^+/NH_3 can out-compete SMX or TMP for free chlorine at pH ~ 8.3. Additional experiments have shown that reactions between combined chlorine and SMX or TMP are extremely slow ($t_{1/2}$ > 1 d), and that chlorine transfer from the respective N-chloro intermediates to free NH_4^+/NH_3 is negligible at acidic pH during a suitable analytical time-frame, thus indicating the applicability of NH_4Cl as a quenching agent under appropriate conditions. Pseudo-first-order kinetics was observed for oxidation of the antibiotics by FAC. Second order kinetic rate constants for all reactions were calculated from observed pseudo-first order constants on the assumption that oxidant concentration remained essentially constant over monitoring periods utilized in experiments performed during this investigation. Solution pH strongly influenced reaction rates. A second order rate model that incorporates speciation of both antibiotic and chlorine oxidant was developed to evaluate the observed pH trend. Consideration of predominant antibiotic species and corresponding reaction rates at specific pH values allowed a preliminary determination of reactive sites within the

antibiotics. Structurally-related compounds that correspond to either the hypothesized reactive or inactive portions of SMX or TMP were also examined in order to verify the proposed site(s) of reaction. LC/MS was utilized to identify reaction products and to assess product evolution during each reaction time course. Product characterization and temporal distribution provided further insight into reaction pathways and mechanisms. Additionally, oxidation of TMP and SMX in real environmental matrices was used to assess applicability of findings based on the above laboratory studies. The results of this study indicate that representative members of two widely-used and environmentally-significant antibiotic classes are substantially transformed to various higher molecular weight and lower molecular weight reaction products in clean systems under conditions simulating chlorination of water supplies during disinfection processes. Maximum reaction rates are observed for each of the compounds under conditions likely to be encountered in water treatment facilities (i.e., pH values between 6 and 8). SMX and TMP were substantially degraded in real wastewater and drinking water samples under conditions applied during disinfection at a municipal wastewater reclamation facility and drinking water treatment facility, respectively. However, neither SMX, nor TMP were significantly degraded by combined chlorine in clean systems over time intervals corresponding to typical disinfection contact times. Kinetic data (for surrogate compounds, as well as TMP and SMX) and structural characterization of reaction products have been utilized here to develop general degradation pathways for the antibiotic classes to which SMX and TMP correspond.

CHAPTER I. CHEMICAL OXIDATION OF FLUOROQUINOLONE ANTIMICROBIALS BY FREE AND COMBINED CHLORINE.

Introduction

As early as 1985, Richardson and Bowron drew attention to the likely presence of pharmaceutical compounds in municipal wastewater effluents and potable water supplies [1]. More recent studies have verified the ubiquity of numerous classes of pharmaceutically active compounds (PhACs) within the aquatic environment [2-4]. Amongst these PhACs, several widely-applied veterinary- and human-use antibiotic classes have been repeatedly detected in concentrations ranging from low ng/L to low μg/L [2, 5-9]. The fluoroquinolones, sulfonamides, and dihydrofolate reductase inhibitors – together with tetracyclines and macrolides – represent several of the most commonly used classes of modern antibiotics, as well as several of the most frequently detected forms of antibiotics in environmental occurrence studies.

Naturally, the regular detection of such antibiotic residues in the water supply lends significance to understanding their fate in natural and engineered aquatic systems. A prior study indicates that aerobic bacterial biodegradation of the fluoroquinolone ciprofloxacin is extremely limited, which would certainly be expected, considering the intended biological effects of antibiotic compounds in general [10]. Accordingly, biological degradation of antibiotic compounds via conventional activated sludge processes in municipal wastewater treatment does not appear to be a major factor in their environmental fate. On the other hand, Wetzstein et al.

[11, 12] have reported extensive degradation of the fluoroquinolones enrofloxacin and ciprofloxacin by hydroxyl radical-mediated enzyme systems characteristic of brown-rot fungi (*sp. Gloeophyllum striatum*). In addition, significant photolytic degradation (by combinations of direct UV photolysis and radical-mediated mechanisms) of fluoroquinolones has been reported [13, 14].

The presence of basic, highly nucleophilic tertiary and/or secondary amino groups within the structures of all fluoroquinolone antibiotics would seem to suggest that they should be quite susceptible to oxidative attack, via ozone, advanced oxidation processes, chlorine, or other oxidants commonly used in water treatment [15, 16]. Observations from a recently completed occurrence study verify these expectations, as various fluoroquinolones (in addition to sulfonamides and trimethoprim) are nearly completely transformed through chlorine and ozone disinfection processes in several municipal wastewater treatment plants within the United States [9, 17]. Aqueous chemical oxidation mechanisms would appear to be efficient means of modifying -- in some cases mineralizing – antibiotics from the fluoroquinolone family.

In several previous studies, the end result of oxidative transformation of fluoroquinolone antibiotics seemed to be either a reduction or elimination in biological activity, namely with respect to antibacterial properties [18, 19]. However, Sunderland et al. [19] found that mixtures of some fluoroquinolone oxidation products do retain a measurable degree of antimicrobial activity. Accordingly, it is important to understand the significance of oxidative treatment processes not only with respect to removal of the parent compound from water supplies , but also with respect to structural modification.

To date, the bulk of observations obtained from chemical oxidation studies (with the exception of those pertaining to photolysis) for the fluoroquinolones have been limited to general assessment of reaction rates or product formation, with little elucidation of reaction mechanisms or degradation pathways. The current investigation was undertaken with the intent of quantifying reaction kinetics and clarifying the reaction pathways involved in oxidative degradation of the environmentally significant (i.e., frequently detected in relatively high concentrations) fluoroquinolone antibiotics (Table I-1) by free available chlorine (FAC) and combined available chlorine (CC) under conditions associated with chlorine-based municipal wastewater and drinking water disinfection processes.

Owing to the presence of acidic and/or basic functional groups in each of the antibiotic structural classes considered here, solution pH was expected to strongly influence the reaction rates between HOCl/OCl$^-$ and the compounds studied. The fluoroquinolone antibiotics all possess two macroscopic dissociation constants, and exhibit speciation relationships as shown in Figure I-1. Consideration of predominant antibiotic species and corresponding reaction rates at specific pH values was intended to provide a preliminary determination of reactive sites within the antibiotics. The structurally-related compound flumequine – corresponding to the inactive portion of ciprofloxacin – was examined in order to verify the proposed site(s) of reaction. Oxidation reactions for three additional fluoroquinolone antibiotics (ofloxacin, enrofloxacin, and lomefloxacin: see Table I-1) were also examined as comparisons to ciprofloxacin. LC/MS was utilized to identify reaction products and to assess product evolution during each reaction time course. Product characterization and temporal distribution provided further insights into reaction

pathways and mechanisms. Additionally, oxidation of ciprofloxacin in real environmental matrices has been used to assess applicability of observations from this study.

Materials and Methods

Standards and Reagents. Ciprofloxacin (CF) hydrochloride, enrofloxacin (EF), ofloxacin (OF), and flumequine (FLU) were obtained from ICN Biomedicals, Inc. (Irvine, California; USA). Lomefloxacin (LF) hydrochloride was purchased from Sigma-Aldrich Co. (St. Louis, Missouri; USA). All standards used for preparation of stock solutions were reagent grade and were used without further purification. All reagent solutions (e.g., buffers, stocks, oxidants, quenching agents) were prepared using Nanopure© purified water. 100 mg/L stock solutions (for use in kinetic experiments) of all compounds were prepared in 10% methanol. 1 g/L stock solutions (for use in LC/MS product characterization studies) of CF were prepared in 50% methanol. Stock solutions were discarded after two months of storage at <5° C.

~7% sodium hypochlorite solutions (in water) were obtained from Fisher Scientific International, Inc. (Pittsburgh, PA; USA) and diluted to ~100 mg/L (free chlorine) stock concentrations for use in kinetic experiments. Free chlorine stocks were periodically standardized iodometrically [20]. N,N-diethyl-*p*-phenylenediamine (DPD) was used through either spectrophotometric (DPD colorimetry) or titrimetric (DPD-FAS) techniques to measure free chlorine residual concentrations after completion of kinetic experiments [20].

Pre-formed chloramine stocks were prepared at pH values of 4.5, 5, 6, 7, 8, 9 in 0.1 M acetate (pH = 5), phosphate (6 = pH = 8), and borate (pH 9) buffers, with modifications to the

methods of Chapin [21]. 302 mg/L solutions of NH_4Cl and 200 mg/L solutions of FAC (prepared from diluted 7% FAC stock and standardized iodometrically) were mixed at 25°C in 1:1 proportion (to produce ~100 mg/L CC solutions, at 2:1 NH_4Cl:FAC molar ratios) under completely-mixed conditions, and allowed to react for no less than four hours, to ensure completion of dichloramine formation at lower pH values. CC stocks were prepared prior to each experiment, temporarily stored at <5°C, and used within 24 hours of generation. CC concentrations were standardized using DPD-FAS titrimetry, to provide information on the distribution of monochloramine and dichloramine within each stock solution.

Kinetics in Buffered Deionized Water Systems. Temperature was maintained at 25°C in all experiments, using a NESLAB RTE-111 (Thermo NESLAB – Portsmouth, NH; USA) recirculating water bath connected to an acrylic water tank. Reaction solutions were partially immersed in the water tank and stirred with Teflon-coated stir bars using a 15-position magnetic stir-plate (Vario-MAG – Daytona Beach, FL; USA). A mercury thermometer was used to verify the temperature set-point within the water tank.

0.01 M acetate (pH 4, 4.5, 5), phosphate (pH 6, 7, 8), and borate (pH 9) buffers were used to maintain pH in all experiments except for those involving reaction of CF and FLU with FAC, for which only 0.01 M phosphate buffers were used. 0.01 M phosphate buffers were found to maintain constant pH relatively poorly at values deviating from the pK_{a1}, pK_{a2}, or pK_{a3} for H_3PO_4 by more than $pK_a \pm 2$, so all experiments conducted subsequent to those involving reaction of CF with FAC were performed using a combination of acetate, phosphate, and borate buffers. Addition of substrate and oxidant to buffered reaction solutions did not alter initial solution pH by more than 0.01 units. pH values of reaction solutions were checked after

completion of each kinetic experiment to verify that solution pH did not vary by more than 0.05 units from starting values. In cases for which final pH deviated by more than 0.05 units from the starting values, the final measured pH was taken as the reaction pH. Free and combined chlorine reaction kinetics were assessed for CF using at least six pH values in the range pH 4 to 9. Kinetics for all other compounds were assessed at pH 4, 7, and 9.

Reactions were initiated by addition of appropriate volumes of 100 mg/L FAC or CC stock (to achieve 11:1 oxidant:substrate ratio for CF, 10:1 for all other compounds) to solutions containing 500 μg/L of substrate and 0.01 M buffer, under completely-mixed conditions, at 25°C. Reactions were monitored by quenching 1-mL samples of each reaction solution at appropriate time intervals and analyzing by HPLC with fluorescence detection. Excess sodium thiosulfate (2:1 $[Na_2S_2O_3]$:$[FAC]_0$, on a molar basis) was used to consume residual chlorine in all reactions.

Free chlorine and total chlorine residual concentrations were measured by DPD colorimetry or DPD-FAS titrimetry [20] at the end of each kinetic experiment in order to verify applicability of pseudo-first order conditions.

Kinetics in Real Water Systems. Water samples taken from a regional wastewater reclamation facility (WRF) and a drinking water treatment plant (WTP) were used to assess the applicability of observed reaction kinetics and temporal product distribution to wastewater and drinking water disinfection, respectively. 2-L samples of secondary wastewater effluent and filter box effluent were collected from the WRF and WTP, respectively. Samples were transported to the laboratory in sealed amber borosilicate bottles with Teflon caps, without preservatives, and stored on ice. The sample from the WRF corresponds to the point in the

plant's treatment train just prior to application of chlorine gas, while the sample from the WTP corresponds to the point in the plant's treatment train subsequent to pre-chlorination by chlorine dioxide (to reduce chlorine demand) and immediately prior to disinfection by chlorine gas. Residual chlorine measurements provided by the plants verified the absence of available chlorine (free or combined) in either sample. These two samples provided representative examples of low ammonia process waters typically subjected to disinfection by application of chlorine oxidants (e.g., ClO_2, NaOCl, Cl_2).

20-mL reaction volumes of each water sample were spiked with 500 µg/L of a given antibiotic and subjected to free chlorine concentrations approximating those utilized by the plant from which they were derived ($C_{FAC,\ WTP}$ = 2 mg/L, $C_{FAC,\ WRF}$ = 10 mg/L), where each plant doses the respective process waters with enough free chlorine to meet the water's chlorine demand and leave a residual of ~ 1 mg/L. Each reaction was followed in the same manner as for the clean water systems described above.

Competition Kinetics. An attempt was made to determine the second-order rate constant for reaction of CF with FAC by use of competition kinetics [22]. Resorcinol (1,3-dihydroxybenzne) was selected as a competitive substrate, based on availability of its second-order constant and its relatively rapid oxidation by hypochlorous acid in aqueous solution (calculated k_{app}'' = 5.4 x 10^3 $M^{-1}s^{-1}$, at pH =7) [23]. Batch reaction solutions were prepared in 0.01 M pH 7 phosphate buffer, using equimolar proportions of resorcinol and CF ([substrate]=1.51 x 10^{-5} M). 1-mL samples of these solutions were taken prior to addition of 1.51 x 10^{-6} M FAC, in order to obtain benchmark unreacted resorcinol signal area (via HPLC). Reactions were allowed to proceed for 1 minute, at which time excess $Na_2S_2O_3$ was added to

quench residual active chlorine in order to prevent subsequent oxidation of resorcinol by combined chlorine associated with N-chlorinated intermediates of CF. 1-mL samples of the quenched reaction solution were then analyzed by HPLC, in order to obtain signal area for resorcinol after addition of FAC. Controls (without CF) were utilized to obtain pre- and post-reaction signal areas of resorcinol in the absence of CF.

LC/MS Product Characterization Studies. 2 mL of 1 g/L CF antibiotic stock was added to 18 mL of 0.01 M pH 7 phosphate buffer to achieve starting concentrations of 100 mg/L. Free chlorine was added to solution from ~7% sodium hypochlorite stock to initiate oxidation at 4:1 (oxidant:substrate) molar ratios. Unquenched 1-mL samples of the reaction mixture were added to autosampler vials for immediate analysis by LC/MS.

HPLC and LC/MS peak matching. CF was added to pH 7 phosphate buffer to achieve 100 mg/L starting concentration as above, and dosed with FAC accordingly. 100 µL volumes of unquenched reaction solutions were separated five times (for a total sample volume of 500 µL) by HPLC, and appropriate fractions were collected (using an ISCO Foxy, Jr. fraction collector) for each major product peak identified in kinetic studies. Dilute fractions were subsequently reconcentrated to approximately 500 µL by evaporating under nitrogen gas at ~50°C and analyzed by LC/MS to verify their identity using their respective m/z values.

Analytical Methods. An Agilent 1100 series HPLC system equipped with a 5 µm particle-diameter, 4.6 mm x 250 mm Zorbax RX-C18 column, a UV/Vis diode-array detector (DAD), and fluorescence detector (FLD) was used to monitor residual concentrations of parent compounds and signal areas of products generated during the course of each oxidation reaction. Depending on the time constraints of each study or on chromatographic behavior of respective

analytes, gradient and isocratic methods were used with varying ratios of 0.04 M H_3PO_4 buffer to pure acetonitrile.

An Agilent 1100 series HPLC system equipped with a 4.1 μm particle-diameter, 2.1 mm x 150 mm Zorbax SB-C18 column, a UV/Vis DAD, and an 1100 series single-quadrupole mass spectrometer was used in the characterization of reaction products. Gradient elutions, using varying proportions of 0.2% (v/v) formic acid to pure acetonitrile, were used to separate product mixtures prior to analysis by electrospray ionization (ESI) at each of two fragmentation voltage settings (80 V and 120 V, for low and high degrees of fragment generation, respectively).

TOC analyses were conducted using a Shimadzu TOC-5050a Total Organic Carbon Analyzer and obtained according to standard methods for determination of non-purgeable organic carbon (NPOC) [20].

Results and Discussion

LC/MS Product Characterization. Mass chromatograms (80 eV fragmentation voltage) corresponding to oxidation products of CF yielded a number of prominent, stable peaks (Table I-2). In addition, a number of transient peaks were identified, which appear to correspond to N-chlorinated (chloramine) intermediates: m/z 352 (1 Cl); molecular ion of m/z 374 (2 Cl); molecular ion of m/z 366 (1 Cl); and m/z 408 (3 Cl). The number of chlorines assigned to each compound was based on relative abundance of Cl isotopic peaks. Classification of these compounds as N-chlorinated intermediates is based primarily on their behavior as transient

peaks in low FAC reaction mixtures (i.e., 4:1 FAC:CF molar ratio or less). Repeated analyses of low FAC reaction solutions (unquenched) showed that the areas these peaks decreased over spans of several hours, giving way to stable dealkylated and/or ring-chlorinated products. In addition, the peaks corresponding to m/z 366, 374, and 408 were isolated through HPLC fraction collection. These peaks – after several hours – exhibited spontaneous rearrangement to stable products with m/z 306, 263 and 297, respectively.

The peak corresponding to m/z 366 (a mono-chlorinated CF molecule) was not observed in great abundance in product characterization studies, though the reaction period of 60 minutes allowed prior to sample analysis by LC/MS allowed extensive time for this intermediate to decay – yielding dealkylated products. However, addition of FAC to CF in under-stoichiometric amounts (1:2 FAC:CF, on a molar basis) led to generation of the m/z 366 intermediate in near exclusivity, in turn indicating that this particular compound is the primary intermediate in reactions between CF and FAC. Addition of higher relative quantities of FAC (i.e., > 1:1 FAC:CF) resulted in generation of additional intermediates – including m/z 374 and 408, suggesting that the decay of m/z 366 to the dealkylated product (m/z 306) is followed very rapidly by N-chlorination of this dealkylated product to yield N-chlorinated intermediates corresponding to m/z 374 and 408 (m/z 306 + 2 Cl (- 2 H) and + 3 Cl (- 3 H), respectively), which – as mentioned above – decay to yield further dealkylated products with m/z 263 and m/z 297.

Based on similarities in reactive moieties amongst all fluoroquinolones (as will be explained below in the discussion of kinetic experiment results) and on the results of Zhang and Huang [24] (which – in a recent study on manganese oxide facilitated oxidation of the fluoroquinolones

CF, EF, OF, LF, and norfloxacin – showed similar distributions of reaction products as those identified here) the product distribution of CF can be taken with reasonable confidence to apply to other compounds within the fluoroquinolone structural class.

Kinetic Studies in Clean Systems. Pseudo-first order kinetic rate constants were determined (when possible) at various pH values in order to assess the effect of pH on reaction rate, and to allow a preliminary assessment of probable reaction sites for the attack of HOCl/OCl⁻ on each compound. Measured values of $\ln(C/C_0)$ were plotted against time (in minutes) in order to obtain the pseudo-first order rate constants (slope = $-k_{app}'$) in units of min^{-1}. An example plot is given in Figure I-2, for oxidation of OF by FAC at pH=4. Linearity of pseudo-first order plots was checked via calculation of linearity coefficients, r^2, which ranged from 0.96 to 1. Each kinetic experiment involving CF was conducted in triplicate. Experiments for all other FQs were conducted in duplicate. 95% confidence intervals were calculated and reported with all rate constants as error bars in relevant graphs.

Second-order rate constants were calculated according to procedures used by von Gunten and Oliveras [25]. Because oxidants (FAC or CC) were utilized in significant excess of substrates (10:1 oxidant:substrate on a molar basis), their total concentration $[FAC]_T$ or $[CC]_T$ can be assumed as constant over the course of reaction studied during this investigation. Thus, division of the measured pseudo-first order constant:

$k_{app}' = k_{app}''[Oxidant]_T$, in units of s^{-1}

by total oxidant concentration yields the apparent second order kinetic rate constant:

$$k_{app}'' = \frac{k_{app}'}{[Oxidant]_T}, \text{ in units of } M^{-1}s^{-1}$$

FLU was not oxidized by FAC during the course of two-hour monitoring periods (results not shown), and has thus been characterized as non-reactive over the experimental durations typical of this investigation.

Kinetic Studies in Clean Systems – CF, LF with FAC. Attempts were made to measure the rate of direct reaction of CF with FAC, using competition kinetics [22], with resorcinol as the competitor. Results indicate that the direct reaction of CF with FAC proceeds at an extremely rapid rate ($k_{app}'' >> 5.4 \times 10^3 M^{-1}s^{-1}$ – which is the measured apparent second order constant for resorcinol – on the basis of complete out-competition of resorcinol for FAC), beyond the reach of analytical techniques available in our laboratory. This is not surprising, given results obtained by the Research Laboratories of the United States Army's Edgewood Arsenal [26-28] in studies on oxidation of triethylenediamine by hypochlorous acid. As observed in that research [26], initial oxidation of triethylenediamine proceeds by way of direct attack by HOCl on either of the molecule's amine nitrogens, followed by hydrolytically-mediated dealkylation (loss of an ethyl group – in the form of two molecules of formaldehyde) to yield piperazine (Figure I-3). The piperazine produced will then react extremely rapidly with HOCl to yield dichloropiperazine, which subsequently decays to yield additional formaldehyde molecules and ethylenediamine via dealkylation (Figure I-3). Hull et al. [27] concluded that reactivity of piperazine must be even higher than that of triethylenediamine, which was observed in a separate study to react completely with hypochlorous acid in less than 5 milliseconds [28]. Selection of a more highly reactive competitor could possibly permit a quantitative determination

of CF reactivity, although undesired side-reactions with N-chlorinated intermediates of CF could possibly result in interferences, thus complicating interpretation of results.

On the basis of structural similarities and consideration of relative nucleophilicity of the N_6 position (see CF, Table I-1) of the piperazine ring (which has been verified as the initial reactive site in fluoroquinolone molecules), LF is assumed to react even faster with FAC than CF. The pK_a value for complete deprotonation of the LF N_6 atom is approximately 8.8, while that for CF is approximately 8.5 (Table I-1), indicating that the N_6 position of LF is more basic (and hence more nucleophilic) than that of CF, and should in turn exhibit a higher propensity toward oxidative attack by FAC. As will be discussed in greater detail below, previous studies have shown a direct correlation between basicity of nitrogen atoms and their reaction rate with chlorine oxidants [15, 29]. In addition, the substitution of a CH_3 group ortho to the N_6 position could also be expected to contribute to increased electron-density at the N_6 position in the LF molecule via inductive effects, also increasing its susceptibility toward electrophilic attack by FAC. The presence of an additional F atom on the aromatic ring to which the piperazine ring is anchored is not expected to contribute to electronic properties of the N_6 position, as separation of the two atoms is quite large, and the aliphatic nature of the piperazine ring prevents any resonance effects from affecting N_6 properties at a distance [30].

Kinetic Studies in Clean Systems – OF, EF with FAC. OF and EF – which contain tertiary-substituted N_6 atoms (rather than secondary-substituted amines, as do CF and LF) – exhibit significantly lower reaction rates (k_{app}'' = 6.43 x 10^3 $M^{-1}s^{-1}$ and 4.69 x 10^2 $M^{-1}s^{-1}$ for OF and EF, respectively, at pH 7) in comparison to CF and LF (for which k_{app}'' is much faster than

5.4 x 10^3 $M^{-1}s^{-1}$, as discussed above). The relative reactivities and pH-dependencies of OF and EF are shown in Figures I-4 and I-5.

Maximum observed reaction rates for OF and EF correspond to intersection of the speciation plot for HOCl and the neutral antibiotic species, as would be expected, based on previous studies of pH-dependent N-chlorination [15, 29]. A decrease in reaction rates is also evident at acidic or alkaline pH values, corresponding to decreases in the primary reactive species (decrease in neutral amino-N at acidic pH, and decrease in HOCl at alkaline pH). A comparison of the observed reactivities for OF and EF indicates that EF reacts approximately one order of magnitude slower than OF. This can most likely be explained by increased steric hindrance to oxidative attack, due to the presence of the larger N-ethyl substituent (EF) at N_6 relative to the N-methyl substituent (OF). The observed trends in pH-dependency provide evidence that attack of the OF and EF structures occurs at the N_6 position of the piperazine ring, which should be much more susceptible to oxidative attack than the N_1 atom (see CF, Table I-1), which is situated immediately adjacent to strongly electron-withdrawing *o*-fluorine substituent, and should in turn exhibit greatly reduced basicity via direct inductive effects.

As postulated by Morris [15], nucleophilicity of nitrogenous substrates should correlate directly with affinity for univalent Cl^+, and thus strongly nucleophilic bases such as piperazine could be expected to exhibit very high reactivities. On the other hand, the presence of additional alkyl substituents on an amino nitrogen results in competition between steric and nucleophilic effects with regard to attack by strong oxidants. Several studies have indicated a general degree of amino reactivity toward hypochlorous acid best illustrated (in order of decreasing reactivity) as: primary>secondary>tertiary [15, 29]. This is of course a very general

trend, which can perhaps be better explained via more specific consideration of the competing effects of nitrogen nucleophilicity and steric hindrance. Positive effects (i.e., increasing susceptibility to oxidative attack by FAC) are derived from increasing nucleophilicity, while negative effects (i.e., decreasing susceptibility to oxidative attack) are typically derived from increasing size of substituent, via steric hindrance.

Abia et al. [29] presented a review of primary, secondary, and tertiary amine chlorination rates, in which direct correlations were observed between degree of nucleophilicity and chlorination rate for each class of N-substituted amines. However, a significant drop in reactivity was noted when moving from primary to secondary to tertiary amino compounds (hence the general trend in reactivities noted earlier in this paragraph). The correlation in reactivity of tertiary aliphatic amines described by Antelo et al. [31] should also apply reasonably well to the case of tertiary alicyclic amines (such as those characteristic of the OF and EF molecules), with the exception of such cases as triethylenediamine, which as noted earlier, exhibits extremely high degradation rates in the presence of HOCl (most likely due to the arrangement of the alkyl substituents in this case, which results in elimination of steric effects typically associated with higher degrees of N-alkyl substitution, while maintaining a high degree of nucleophilicity).

Additionally, with regard to reaction pathways, it is clear from numerous studies related to chlorination of tertiary amines that existence of a trialkyl-N-chloroammonium ion intermediate [28, 32] derived from OF or EF should be quite fleeting. Such an intermediate should decay extremely rapidly (on the order of milliseconds) to yield a mono-dealkylated amine [26-28, 32]. The relative kinetics associated with degradation of a parent tertiary amine (using triethanolamine as an example) can best be illustrated as follows [26]:

$$(HOCH_2CH_2)_3N: + HOCl \overset{slow}{\rightleftharpoons} (HOCH_2CH_2)_3N^+Cl$$

$$(HOCH_2CH_2)_3N^+Cl + HOCl \xrightarrow{fast} (HOCH_2CH_2)_2NH + 2CH_2O + Cl^-$$

Furthermore, as noted by Ellis and Soper [32] – for the example of triethylamine oxidation by hypochlorous acid – formation of the triethylchloroammonium cation is relatively slow at acidic pH (reaction completion within 30 min.), while decay of the cation to secondary amine is relatively rapid. On the basis of these previous findings, the trialkylchlorammonium intermediates of OF and EF would not be expected to interfere with determination of rate constants by means of the quenching method utilized in this investigation. This expectation has been verified by a comparison of HPLC chromatograms for unquenched and quenched reaction solutions containing FAC and EF at a 2:1 FAC:EF ratio, after respective reaction times of 5 minutes. No difference in peak areas corresponding to EF (or oxidation products) was noted upon addition of $Na_2S_2O_3$ to the reaction solution. This result is assumed to apply to OF, as well, on the basis of structural similarities to EF, as well as on the observed rates of oxidation (Figures I-4, I-5), which qualitatively indicate much more rapid degradation of OF by FAC (relative to EF). In turn, we have concluded that experimental results obtained during this investigation for oxidation of OF and EF by FAC do correspond to direct oxidation of these compounds, and have not been influenced by regeneration of parent substrate (OF or EF) from reduction of transient N-chlorinated intermediates.

Kinetic Studies in Clean Systems – Decay of CF and LF Primary Intermediates. As shown in Figure I-6a & b, the quenching technique utilized to follow degradation of CF by FAC was found to result in regeneration of CF from a chloramine intermediate, namely through

reduction of the intermediate to release a free Cl⁻ ion and yield parent amine (i.e., CF), as will be discussed below. Comparison of experimental controls with the unquenched reaction solution containing CF and FAC (2:1 FAC:CF on a molar basis) at less than 10 minutes reaction time indicates rapid direct oxidation of CF by FAC, and significant loss of parent compound (CF). Chromatograms obtained via HPLC analysis of unquenched samples indicate the formation of two late-eluting peaks at t=21.472 minutes and t=22.017 minutes (Figure I-6a), which – according to LC/MS analyses and peak matching studies – correspond to m/z 366 (1 Cl) and m/z 408 (3 Cl), respectively. As mentioned earlier, chlorination of CF at a molar ratio of 1:2 FAC:CF leads only to generation of m/z 366, while the presence of additional FAC leads to formation of m/z 408. These two peaks both represent intermediates in the degradation of CF by FAC. m/z 366 corresponds to mono-chlorinated CF, presumably with Cl substitution at N_6 (Figure I-7a) of the piperazine ring, while m/z 408 likely corresponds to a tri-chlorinated CF degradate (Figure I-7b), with loss of an ethylene moiety from the piperazine ring.

The intermediate nature of these peaks has been ascertained via their transient nature in chromatograms, within which their signal areas decrease slowly within one hour of initial FAC addition to CF. The conclusion that they are N-chlorinated, rather than ring-Cl-substituted (e.g., on CF's benzene ring), is supported by their disappearance from reaction solutions to which strong reductant (e.g., $Na_2S_2O_3$) is added. Ring-Cl-substitution products of CF oxidation by FAC do not appear to be affected by the presence of reducing agent, as significant quantities of two mono-substituted ring-chlorinated reaction products (m/z 297 and m/z 340) have been identified even in quenched samples.

Addition of reducing agent to the sample solutions described above results in disappearance of the two intermediates - m/z 366 and m/z 408 – accompanied by reappearance of the parent CF (m/z 332) and generation of the product m/z 306 (Figure I-6b). m/z 306 corresponds to CF after loss of an ethylene group from the piperazine ring (structure provided in the later discussion on reaction pathway). CF can be regenerated from the m/z 366 intermediate after losing one chlorine; while the m/z 306 degradate appears to be generated by dechlorination of the second intermediate (m/z 408). This is supported by reduction of solutions containing different ratios of FAC:CF. Reduction of a 1:2 solution (containing m/z 366 in near exclusivity) yields almost exclusively CF, while reduction of a 2:1 solution (containing m/z 408, in addition to m/z 366) yields a much greater proportion of m/z 306. Noting that CF and the degradate corresponding to m/z 306 are both stable in the presence of $Na_2S_2O_3$, no additional products would be expected from the reaction of the two respective N-chlorinated intermediates with reducing agent. Dennis et al. [26] have also observed regeneration of piperazine upon addition of $Na_2S_2O_3$ to aqueous solutions containing *p,p*-N-dichloropiperazine, and made no mention of the generation of additional compounds by reduction of this chloramine.

In turn, we conclude here that reduction of the N-chlorinated compounds results only in regeneration of their respective un-chlorinated analogues. In addition, the extremely rapid reaction rate for direct oxidation of CF by FAC indicates that no unreacted (i.e., free) CF should be present in unquenched reaction solutions after 5 minutes (corresponding to the first sample point in CF kinetic studies). Thus, even though we are not able to directly follow oxidation of CF by FAC, we are able to monitor degradation of the primary N-chlorinated intermediate (the stability of which indicates that their degradation represents the rate-limiting

step in oxidation of CF by FAC) by converting it to CF and following residual CF concentration in quenched reaction solutions. Furthermore, we have been able to follow degradation of additional N-chlorinated dealkylated products (corresponding to free amines with m/z 306, 340, 263, and 297) resulting from decay of the primary intermediate by monitoring changes in each their signal areas over time, as will be discussed below. Pseudo-first order constants have been determined for decay of the CF intermediate (m/z 366) at various pH values. Decay of the CF intermediate is likely to be mediated primarily by hydrolysis, as indicated by studies conducted by Dennis et al. [26] – in which decay of *p,p*-N-dichloropiperazine was not enhanced upon addition of greater concentrations of hypochlorous acid, and as indicated by experiments conducted as part of this study – which indicate no statistically significant change in N-chlorinated intermediate decay rate across a range of FAC:CF ratios from 10:1 to 40:1. Thus, pseudo-first order kinetics is more applicable than second order kinetics in this case (assuming H_2O to be the primary reactant with N-chlorinated CF intermediate). Figure I-8 summarizes the results of pH-dependent kinetic studies for decay of the primary CF intermediate, plotted with speciation of the parent antibiotic.

Kinetic experiments involving oxidation of LF by FAC (Figure I-9) produced results quite similar to those involving CF. Based on consideration of structural similarities – the only major difference being substitution of a CH_3 group on the LF piperazine moiety – a conclusion can be made that the reaction rate of LF with FAC will either be equivalent to or higher than for CF (the possibility of greater reactivity being attributed to inductive effects derived from the electron-donating tendency of a CH_3 substituent ortho to the reactive nitrogen, N_6). This conclusion is also supported by the greater basicity of the N_6 nitrogen from LF, compared to

that of CF (pK_{a2} = 8.82 for LF, as compared to pK_{a2} = 8.49 for CF). Furthermore, LF would be expected to yield the same form of intermediate as CF – namely a mono-N-chlorinated intermediate, which should intuitively exhibit similar degradation behavior and produce a similar set of degradates. The relatively higher degradation rate of the LF intermediate (compared to the CF intermediate) can perhaps be explained through a brief consideration of experimental observations obtained for oxidation of 1-chloroaziridines by hypochlorous acid. The chloroaziridine structure, upon oxidation by hypochlorous acid or chlorine yields an intermediate of the type shown in Figure I-10a. The decay of the 1-chloroaziridine intermediate – theorized to proceed through a dealkylated diiminium cation – has been shown to yield a combination of alkyl ketones and inorganic chloramines, similar to the decay of cyclic aliphatic bases such as piperazine and triethylenediamine [33]. Gassman and Dygos [33] studied solvolytic decay (in MeOH and in H_2O) of a series of N-chlorinated methyl substituted aziridines (Figure I-10b,c) in comparison to an unmethylated N-chloroaziridine. Decay rates of the chloroaziridine intermediate increased with addition of methyl groups at the 2 or 3 positions on the aziridine ring, presumably due to a corresponding increase in stabilization (for addition of methyl substituents) of the transition state leading to dealkylation, after heterolytic cleavage of the N-Cl bond to generate (presumably) a nitrenium cation. By analogy, substitution of a methyl group at the ortho position on the LF piperazine ring might be expected to stabilize the transition state leading to dealkylation of the piperazine moiety after addition of Cl to the N_6 position, thus leading to an general increase in the rate of solvolysis (here hydrolysis) of the N-chlorinated LF intermediate, relative to that of the CF intermediate.

One additional note concerning the apparent pH-dependency of the N-chlorinated intermediate decay for both CF and LF should be addressed here. While the pH-dependency shown in Figures I-8 and I-9 is relatively small, it is evident, and it appears to bear some relationship to the speciation of the parent compounds. A direct correlation between the speciation characteristics of parent amines (CF and LF) and the decay rates of their respective N-chlorinated intermediates might not be expected if the decay process involved additional attack by FAC, on the basis of previous experimental observations regarding the effect of N-chlorination on pK_a of the amino group [34] – which results in significant lowering of the substituted nitrogen's pK_a value, and would in turn result in a strong decrease in susceptibility to further chlorination. However, if the decay process is primarily hydrolytic – which is certainly possible, considering the results obtained by Dennis et al. [26] for hydrolytic decay of *p,p*-N-dichloropiperazine, as well as the results obtained in the current investigation (indicating spontaneous dealkylation of the isolated product corresponding to m/z 366 in the presence of water) – a correlation between pK_a of the parent amine (CF or LF) and rate of chloramine decay could be expected. In fact, such a correlation has been noted by Hauser and Moore [35] in a study on decay of ring-substituted N-chlorinated benzaldimines, in which a direct correlation was observed between hydrolytic chlorimine decay rates and pK_a values of the parent imines.

Several additional research groups have noted trends corresponding to base-catalyzed or acid-retarded solvolytic decay of N-chlorinated intermediates. Neale and Walsh [36] observed a decrease in rate of solvolytic dealkylation of N-chloro-di-N-butylamine to yield the intermediate N-chloro-mono-N-butylamine intermediate for increasing concentration of acid, in

the absence of an initiator such as light, and attributed the decreased rate to an increase in the protonation of the N-chlorinated secondary amine, albeit under relatively strong acid conditions. Additional investigations into alkaline decomposition of inorganic chloramines have shown that in the presence of excess OH^-, NH_2Cl may react to form hydroxylamine NH_2OH as an intermediate, followed by reaction with an additional NH2Cl molecule to yield the diimine – HN=NH [37]. The presence of a transient product ion in CF reaction mixture chromatograms, corresponding to m/z 352, with an isotopic peak at m/z 354 – corresponding to one Cl, provides possible support for such a pathway (Figure I-11).

Interpretation of the data generated by oxidation of LF, OF, and EF, and FLU leads to several conclusions:

(1) The piperazine ring is the reactive moiety in oxidation of FQs by FAC. The carboxylic group and heterocyclic aromatic ring of the FQ structure do not participate appreciably in oxidation of fluoroquinolones by free chlorine, as indicated by the stability of FLU when subjected to chlorination.

(2) Oxidation of the fluoroquinolone structure is initiated through attack of the N_6 atom of the piperazine ring, as indicated by observed relationships between FQ structure and reactivity with FAC. Extremely high rates were observed for reaction of free chlorine with CF and LF – which exhibit secondary substitution of N_6. Much lower reaction rates were observed for OF and EF – which both exhibit tertiary substitution of N_6. This correlates relatively well with expected relationships between reactivity with FAC and degree of N-alkyl substitution. Addition of a tertiary alkyl

substituent to a secondary amine typically results in significant reduction of reactivity toward FAC.

(3) Rate constants for the fluoroquinolones OF and EF are dependent on antibiotic and oxidant speciation. More specifically, EF and OF exhibit a pronounced decrease in reactivity with OCl⁻ relative to HOCl, and their cationic forms appear to be much less reactive than their neutral and anion forms. This would also be expected for direct oxidation of CF and LF by FAC – on the basis of structural similarities. However – due to analytical limitations (as mentioned earlier) – we were not able to verify the expected pH-dependency for either CF or LF.

(4) Overall degradation pathways for fluoroquinolones are kinetically limited by the decay of primary mono-N-chlorinated intermediates.

(5) Initial degradation of FQs containing tertiary-substituted amino groups (e.g., OF, EF) is expected to proceed through the trialkylchloroammonium intermediate, after which oxidation pathways should closely parallel those of FQs with only secondary-substituted amino groups (e.g., CF, LF).

Kinetic Studies in Clean Systems – CF with CC. Experimental results for the reaction of CC with CF (Figure I-12) indicate a number of similarities with the results discussed above for FAC. Several conclusions can be made regarding the reaction of CF with CC:

(1) CF is degraded by reaction with both monochloramine and dichloramine species. The pH range studied included values at which both of these chloramines species predominated (dichloramine at low pH, monochloramine at high pH), as verified by

chloramine and CC measurements obtained via DPD-FAS titrimetry. The observed decay rates of the primary CF intermediate at pH values ranging from 4.5 to 9 are evidence of the participation of both oxidant species in the reaction with CF (Figure I-12).

(2) Observed rates of N-chlorinated intermediate decay by CC are generally slower than those for reaction with FAC, but are all within the same order of magnitude. This could be due to the presence of free NH_4^+ during CC experiments, as pre-formed chloramines were prepared using an excess of ammonia, in order to improve oxidant stability. As described in an earlier study conducted by Yoon and Jensen [38], rates of chlorine transfer from representative substrates such as glycine, glycylglycine, and methylamine to free ammonia are appreciable, though approximately two orders of magnitude lower than for transfer in the opposite direction (i.e., from monochloramine to organic substrates). The transfer of active chlorine from N-chlorinated CF intermediates to NH_4^+ has been verified in our investigations through treatment of pre-chlorinated solutions of CF with NH_4Cl, which led to a decrease in the primary intermediate (m/z 366), and a corresponding increase in signal area for free CF.

The results obtained here are significant in their identification of CF's susceptibility to oxidative attack by NH_2Cl and $NHCl_2$, which are commonly thought of as relatively weak oxidant species. This would be expected from a consideration of the relative oxidation strengths of the four oxidant species considered in this investigation, where order of oxidant strength follows the trend $HOCl>OCl^->NHCl_2>NH_2Cl$ [39]. As monochloramine is the weakest oxidant in this chain, it should in turn yield the lowest reaction rates for reaction of CF with CC. The fact that

NH_2Cl is capable of oxidizing CF further indicates that free CF is unlikely to survive any form of water treatment involving the use of chemical oxidants. An explanation for the observed decrease in intermediate degradation rate at pH 9 is not known at this time.

Kinetic Studies in Real Water Systems. Kinetic experiments were conducted in water samples obtained from the WRF and the WTP in order to determine the degree of accuracy with which kinetic calculations based on laboratory results could be applied to actual water treatment systems. Water quality data are provided for each sample in Table I-3. Reactions were studied in both wastewater and drinking water process samples to gauge the relative effect of competing chlorine scavengers and other complicating factors on FQ intermediate decay rates in systems for which chlorine disinfection is likely to be used in current water treatment practice. Decay rates of the primary CF intermediate (m/z 366) closely paralleled those predicted by a pseudo-first order model (Figure I-13), utilizing estimated pseudo-first order rate constants obtained via interpolation of the kinetic results observed in clean systems.

Taken into context with additional kinetic results for OF, EF, and LF, this provides evidence that fluoroquinolones might be expected to out-compete chlorine-demanding compounds and natural organic matter for FAC in typical wastewater and drinking water disinfection scenarios. The rate at which the CF intermediate decayed in real water samples indicates that substantial decay of this compound could also be expected for residence times typical of wastewater disinfection chambers (10-30 minutes) and drinking water clearwells (1-24 hours).

In light of the relatively high concentrations of antibiotics utilized for kinetic studies in this investigation, however, additional experiments utilizing more realistic in situ concentrations could

provide a more certain conclusion in this regard. On the other hand, support for these experimental observations can be provided at full-scale by comparing to results obtained from a recently completed occurrence survey of antibiotic compounds in municipal wastewaters of the United States [9, 17], which showed substantial decreases in aqueous concentrations of fluoroquinolone antibiotics (CF, EF, OF, and norfloxacin) after application of chlorine disinfection. Further study of the kinetics associated with direct oxidation of OF, EF, or other FQs possessing tertiary-substituted N_6 atoms is warranted, as data is not available for these reactions at the present time.

Reaction product distributions associated with oxidation of CF in wastewater or drinking water matrices also parallel those previously obtained for D.I. water systems (discussed below). CC oxidation of CF in real water was not evaluated in this study, primarily because the relative reaction rates associated with FAC oxidation in clean water and real water systems were found to be so similar, indicating that excess chlorine-demanding materials in wastewater or drinking water systems do not significantly alter the overall rate of chloramine intermediate decay.

Proposed Reaction Pathways for CF and FAC, CC. Expected reaction pathways have been developed utilizing a combination of all kinetic data, substructure studies, and product characterization analyses discussed to this point. Figure I-14 depicts the evolution of major products identified by peak matching, using data obtained via HPLC and through LC/MS studies for CF. As a consequence of the quenching technique utilized in monitoring decay of the CF intermediates, plots corresponding to each of the primary degradates (m/z 306, 263, 340, and 297) actually correspond to the abundance of the respective N-chlorinated variants present at the time of quenching. The presence of such a large excess of FAC (11:1 FAC:CF, on a

molar basis) under the reaction conditions utilized for kinetic experiments should preclude the existence of any free amines in solution during the monitoring periods studied in this investigation. Any free amine would be expected to react relatively rapidly with FAC, thus yielding an N-chlorinated derivative. In turn, the data provided in Figure I-14 can be taken to represent the temporal distribution of N-chlorinated oxidation products of each free amine, which also provides snapshots of the relative abundance of each free amine structure at given points in time.

Expected degradation pathways for FQs (developed through the use of kinetic and product characterization data) are presented in Figure I-15. Due to the deactivating effect of the electron-withdrawing fluorine substituent and additional substitution by Cl, the stable product of m/z 297 is expected to represent a probable terminus in the degradation of CF and other fluoroquinolones, and would thus be expected to accumulate in natural and engineered systems. The gradual increase in signal corresponding to this particular product over time (Figure I-14), while other intermediate products plateau or decrease in abundance, would seem to support this conclusion. Furthermore, oxidation of the remainder of the quinolone structure by FAC is extremely slow if it occurs at all, as shown by stability of FLU in the presence of FAC over extended periods of time. Further degradation of the structure corresponding to m/z 297 has been shown to occur via oxidative attack on the aromatic system or carboxyl group via photolytic or peroxidase-mediated processes [11-14], resulting in moderate rates of mineralization in some cases [11, 12]. Thus, the action of peripheral oxidation systems in environmental systems cannot be discounted in modeling the ultimate fate of FQ antimicrobials.

In the case of OF and EF, it is not clear whether dealkylation of the tertiary amine would occur preferentially at the exocylic N-C linkage or at one of the heterocyclic N-C linkages. As Dennis et al. [26] have observed, oxidative fragmentation of a secondary amine such as piperazine occurs by cleavage of the heterocyclic C-N bonds, while fragmentation of compounds such as N-ethylpiperidine or N-(2-hydroxyethyl)-piperidine (both lacking a second heterocyclic nitrogen) occurs at the exocyclic C-N bond, yielding the secondary amine (piperidine). Dealkylation of OF and EF could presumably occur by either of these two possible pathways (Figure I-15).

Products identified by LC/MS for the reactions between CC and CF are the same as those detected for reactions involving FAC. Thus, similar degradation pathways are expected to apply in each case.

Conclusions

The fluoroquinolone antibiotics CF and LF undergo extremely rapid transformation to N-chlorinated intermediate compounds upon exposure to hypochlorous acid at pH values ranging from mildly acidic to mildly alkaline (pH 4 to 9), which includes the range of pH likely to be encountered under conditions typifying municipal wastewater and drinking water disinfection processes. The mono-N-chlorinated intermediate of CF (m/z 366) is apparently relatively stable (15 min. $< t_{1/2} <$ 60 min.), even in the presence of a large excess of oxidant species. Decay of this intermediate in turn appears to represent a rate-limiting step in the overall degradation of CF. Product characterization by LC/MS analyses indicates the formation of a number of

products, represented primarily by four major degradates corresponding to m/z 263, 297, 306, and 340 (corresponding to full or partial dealkylation of the piperazine ring, and – in two cases – substitution of Cl on the quinolone structure's aromatic ring). Based on observations recorded in previous studies, and on structural similarities, degradation pathways for LF are expected to be quite similar. The FQs OF and EF undergo much slower initial oxidation by HOCl – presumably due to steric hindrance of HOCl attack on the N_6 piperazine nitrogen due to the presence of tertiary N-alkyl groups – to yield (theoretically) highly transient N-trialkylchloroammonium cation intermediates, which undergo rapid hydrolysis to further yield a series of dealkylated products similar to those produced by CF. Subsequent degradation of OF and EF is expected to generate oxidation products similar to those identified in CF oxidation experiments.

Table I-1: Selected Model Compounds and Associated Structures

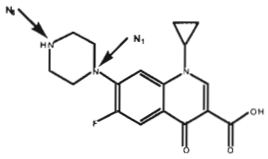
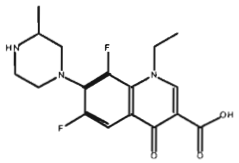
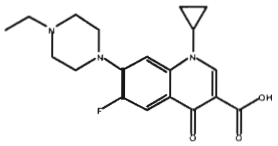
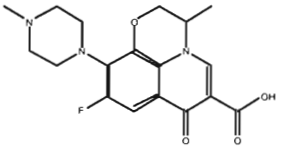
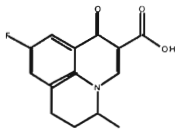
Compound	Structure	Molecular Weight	pK_a
Ciprofloxacin (CF)		331.35	pK_{a1}=6.43, pK_{a2}=8.49 [40]
Lomefloxacin (LF)		351.35	pK_{a1}=6.75, pK_{a2}=8.82 [30]
Enrofloxacin (EF)		359.39	pK_{a1}=6.06, pK_{a2}=7.7 [40]
Ofloxacin (OF)		361.37	pK_{a1}=6.29, pK_{a2}=8.13 [40]
Flumequine (FLU)		261.25	pK_{a1}=6.42 [41]

Table I-2. Oxidation products of reaction between FAC and CF, as analyzed using LC/MS (80 eV fragmentation voltage unless noted otherwise).

#	RT (min)	$M+H^+$	Compound	m/z Fragments
1	10.011	364	Substituted CF	**364** (366, 368, 370)
2	11.11	296	Dealk., Sub. CF	**296** (298, 300), 279 (19%, 281), 253 (53%, 255), 239 (13%, 241)
3	11.401	306	Dealkylated CF	**306**, 288 (17%), 271 (10%), 245 (24%)
4	12.348	332	CF	332, 274 (8%)
5	12.647	340	Dealk., Sub. CF	**340** (342, 344), 332 (12%), 322 (11%, 324), 297 (5%, 299), 265 (5%, 267), 239(6%, 241)
6	12.932	366	Sub. CF	366 (31%, 368), **340** (342), 330 (47%, 332), 322 (10%, 324), 282 (8%, 284)
7	13.632	356	Dealk., Sub. CF	366 (20%, 368), **356** (358, 360), 306 (7%, 308)
8	16.825	263	Dealkylated CF	**263**, 245 (27%)
9	18.647	302	Dealkylated CF	**302**, 289 (10%), 284 (14%)
10	18.974	298	Dealk., Sub. CF	302 (30%, 304), **298** (300), 280 (37%, 282), 261 (22%)
11	19.914	297	Dealk., Sub. CF	**297** (299), 279 (32%, 281)
12	20.433	340	Dealk., Sub. CF	**340** (342), 330 (34%, 332), 322 (19%), 300 (11%), 297 (19%, 299), 287 (17%, 289)
13	24.065	342	Dealk., Sub. CF	**342** (344, 346), 306 (22%, 308)
14	24.691	352	Diimine CF	**352** (354)
15	25.222	374	Dealk., Sub. CF	**374** (376, 378), 352 (15%, 354), 340 (14%), 250 (15%)
16	26.284	374	Dealk., Sub. CF	**374** (376, 378), 364 (20%, 366, 368), 293 (8%), 193 (11%)
17	26.917	366	N-Chloro CF	**366** (368), 245 (6%)
18	28.932	408	Dealk., Sub. CF	**408** (410, 412), 400 (13%, 402), 390 (6%, 392)

Table I-3. Water quality data – wastewater and drinking water samples

Water Quality Data	pH[a]	Alkalinity (mg/L)[b]	NH_3 (mg NH_3-N/L)[c]	TOC (mg/L)
WRF	7.33	120	<MDL	14.0
WTP	6.60	13	<MDL	1.25 [b]

[a]Measured in laboratory, [b]Measured by plant personnel, [c]Measured by plant personnel and verified in laboratory, [d]First measure for wastewater sample obtained on 2/11/03, second for 2/13/03, *MDL=0.12 mg NH_3-N/L

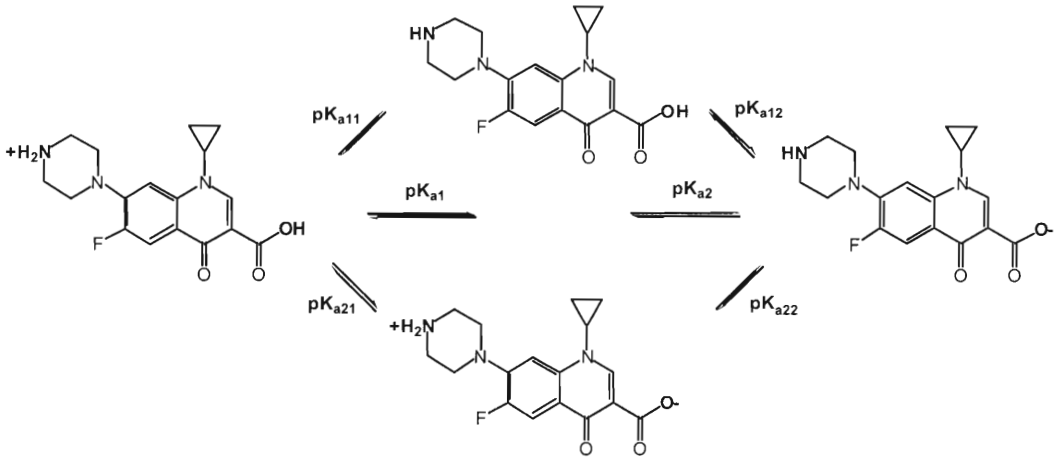


Figure I-1. Speciation of CF (and other fluoroquinolones)

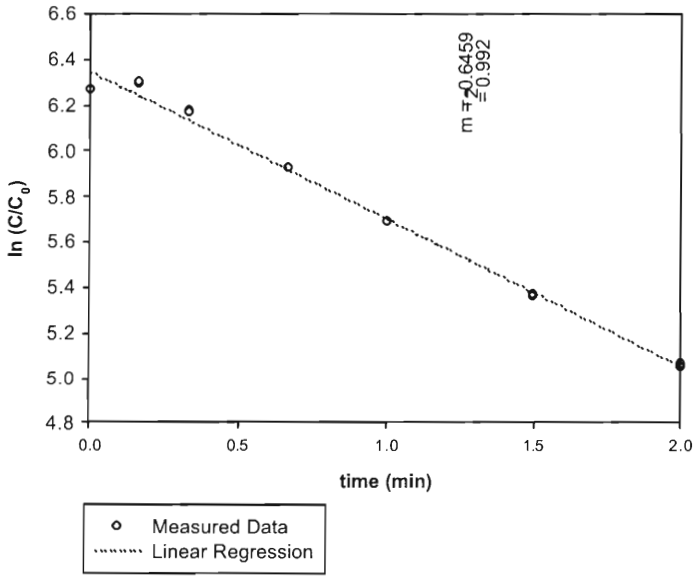


Figure I-2. Determination of pseudo-first order constant for OF decay at pH 4 in 0.01 M phosphate buffer, C_{OF} = 0.5 mg/L, C_{FAC} = 1.07 mg/L, 10:1 oxidant:substrate molar ratio

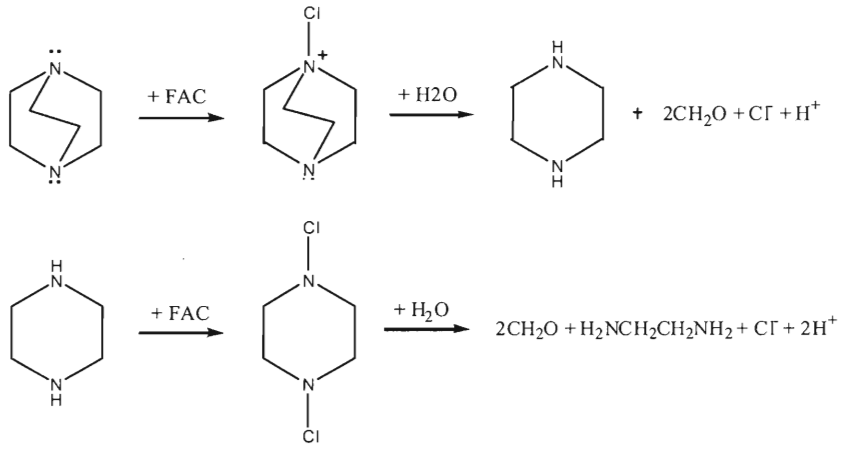


Figure I-3. Degradation pathway for reaction of triethylenediamine with hypochlorous acid [27]

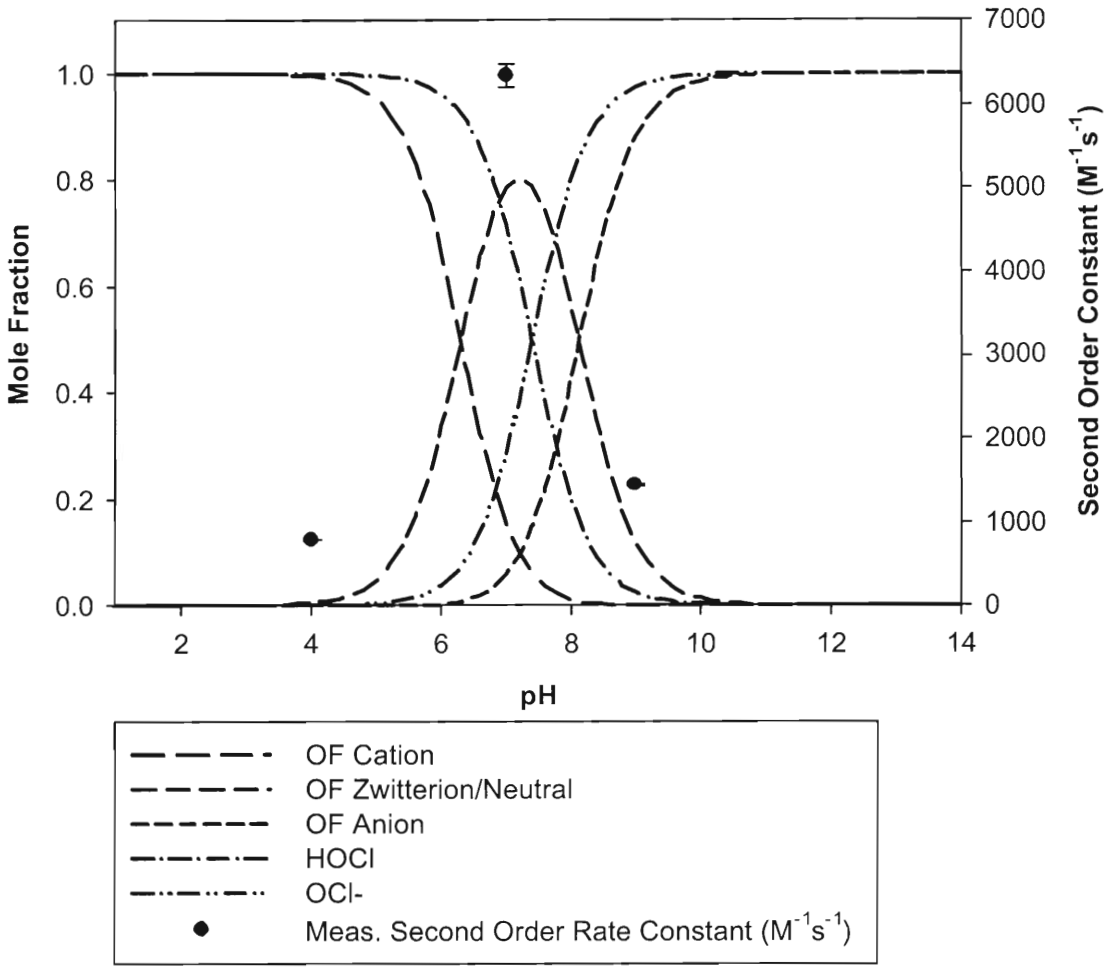


Figure I-4. pH-dependency of apparent second order rate constant for reaction of OF with FAC

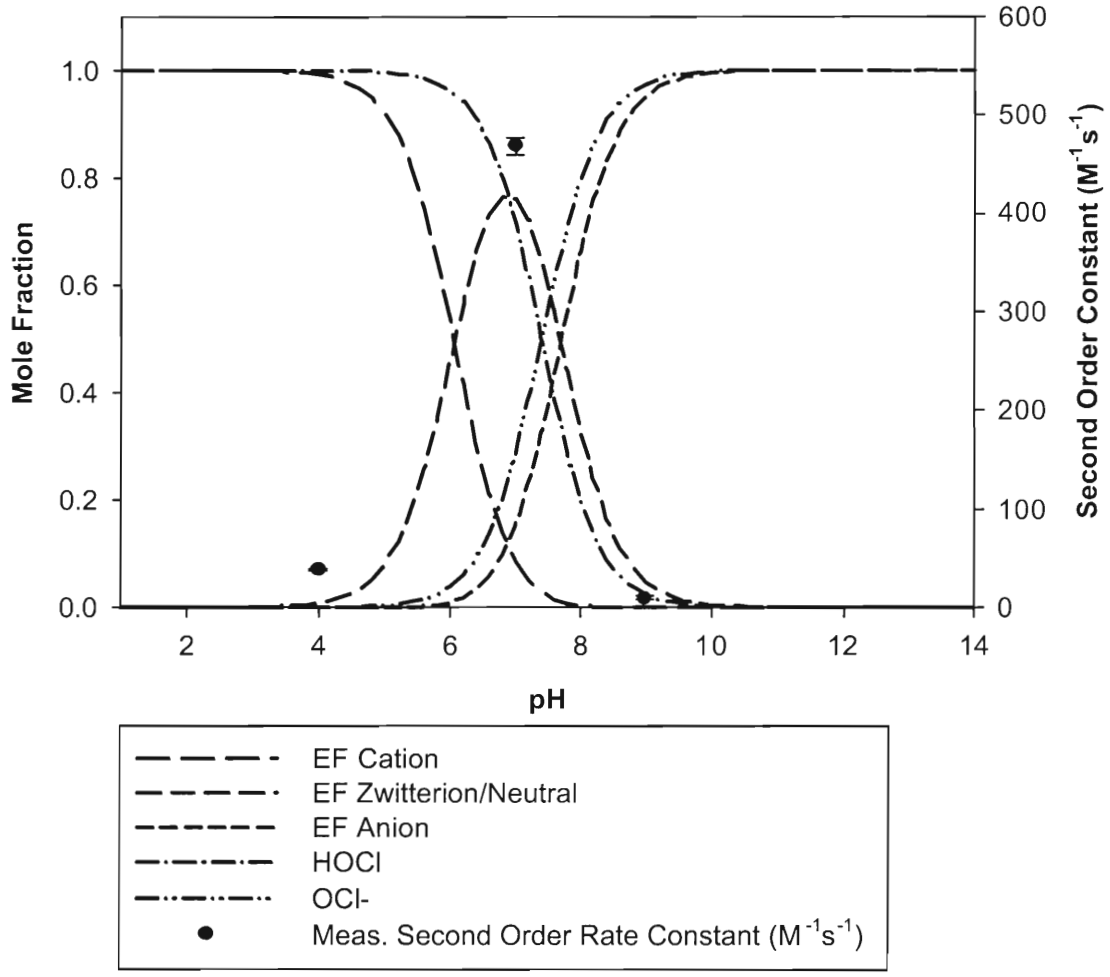


Figure I-5. pH-dependency of apparent second order rate constant for reaction of EF with FAC

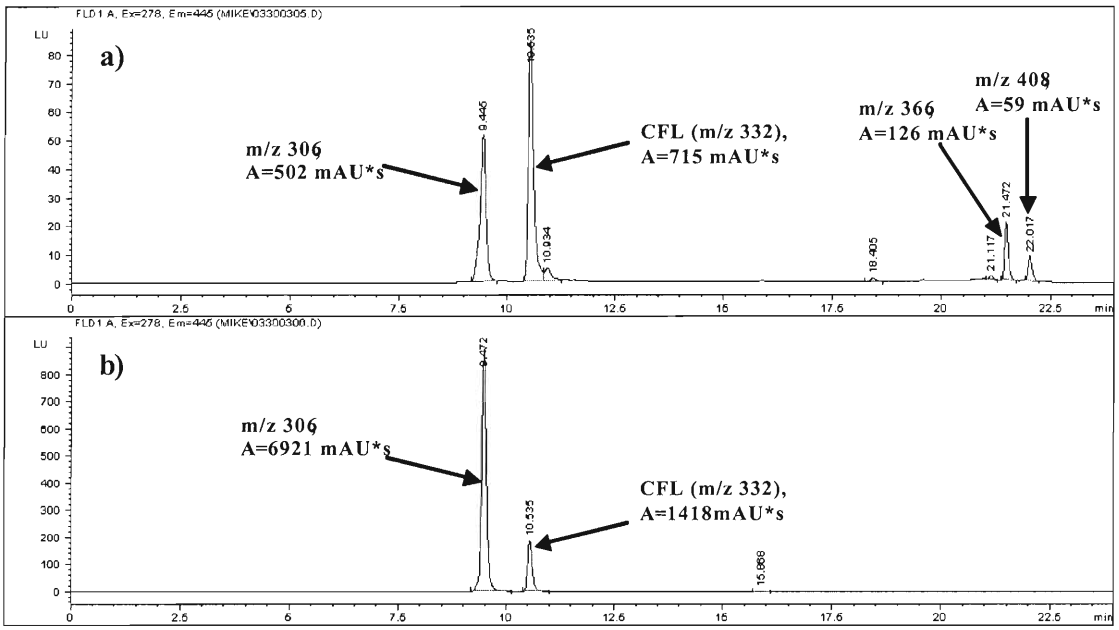


Figure I-6. Chromatograms for unquenched and quenched CF/FAC reaction solutions at t=8.5 min: a) unquenched, and b) quenched – addition of excess sodium thiosulfate

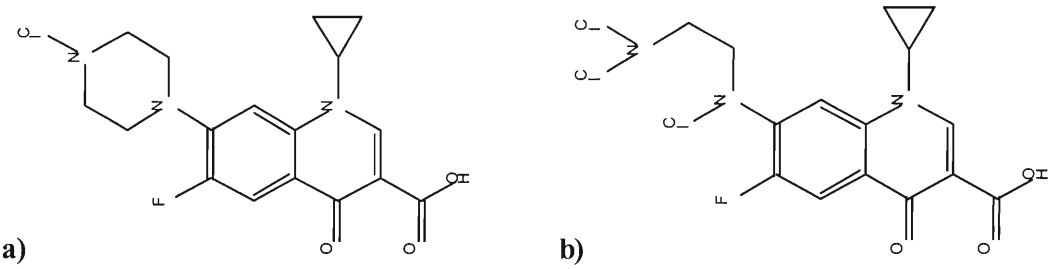


Figure I-7. Proposed structures of two primary N-chlorinated intermediate compounds generated by reaction of CF with FAC: a) m/z 366, b) m/z 408

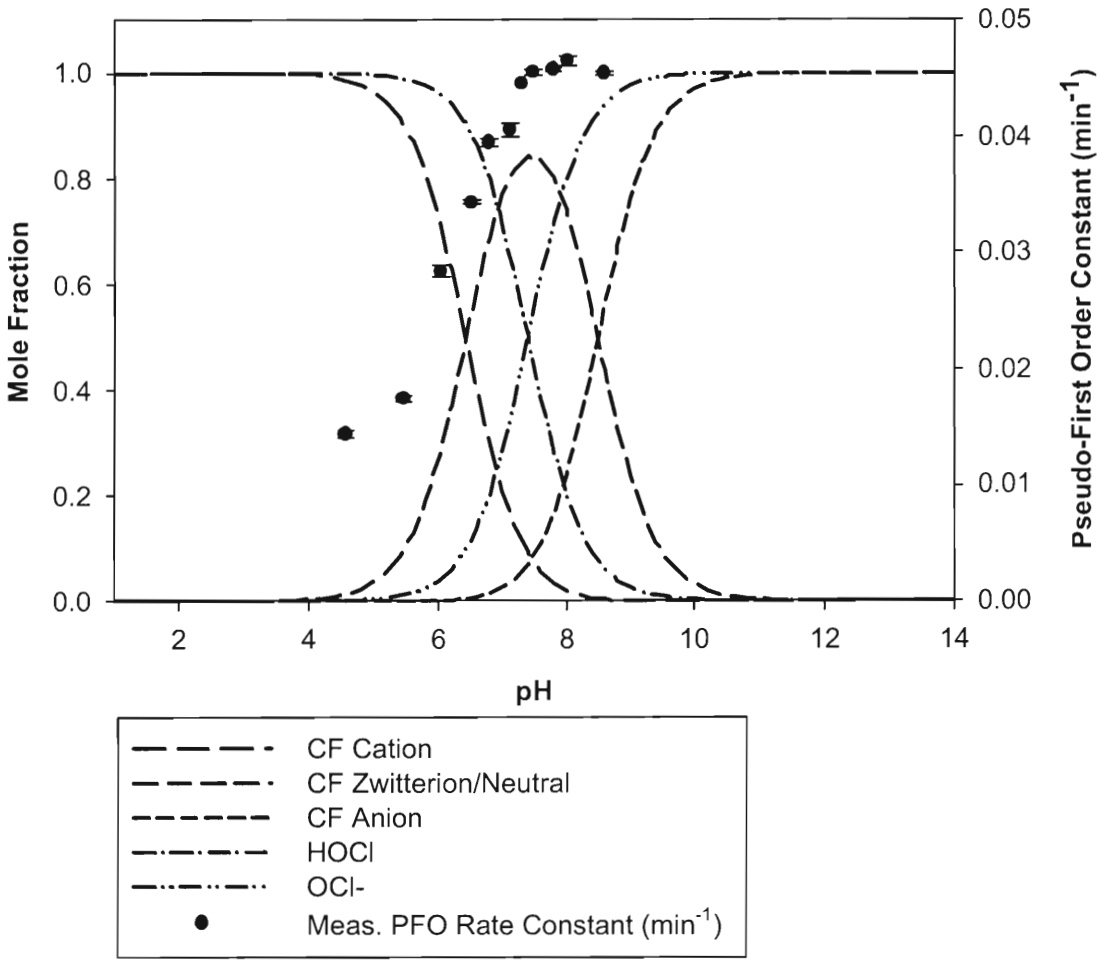


Figure I-8. pH-dependency of apparent pseudo-first order rate constant for decay of N-chlorinated CF intermediate (m/z 366) in the presence of FAC

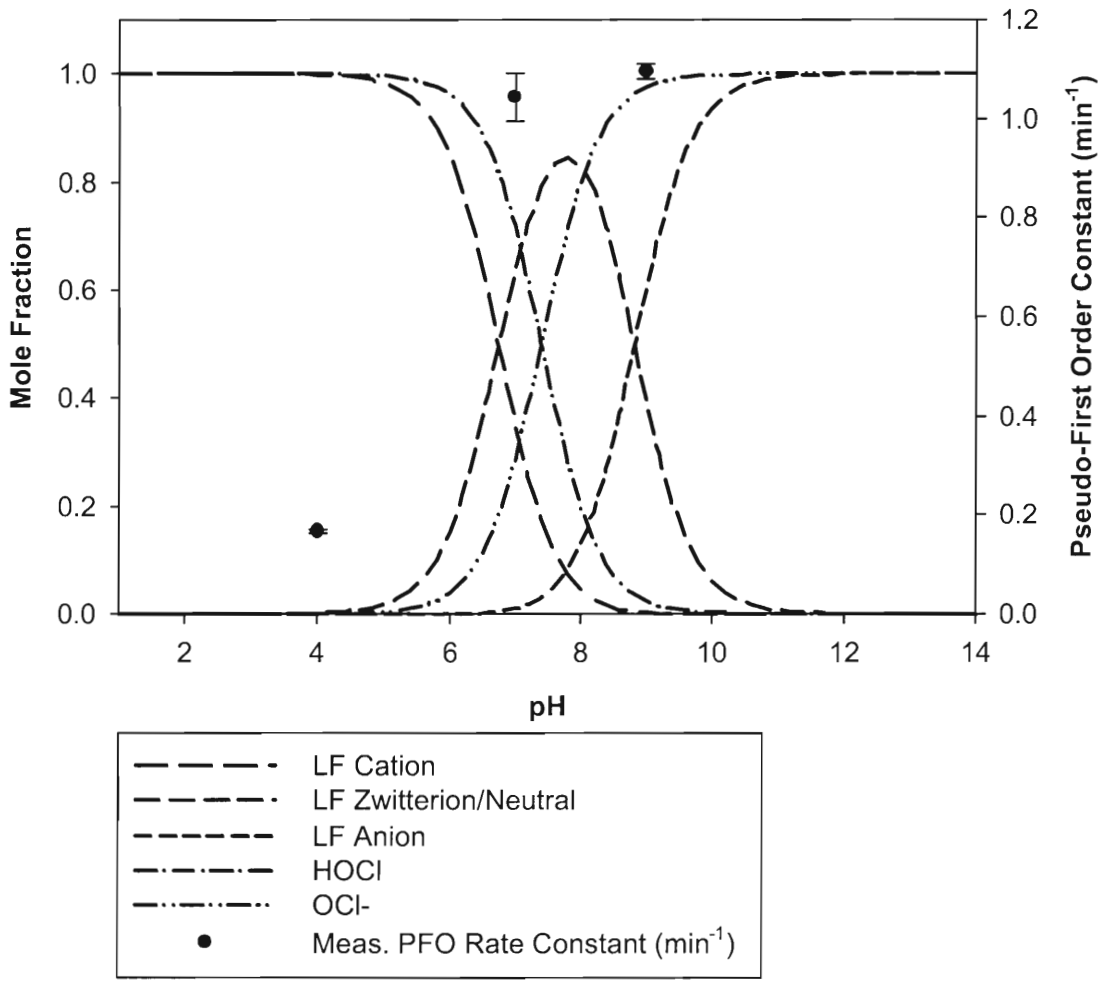


Figure I-9. pH-dependency of apparent pseudo-first order rate constant for decay of primary N-chlorinated LF intermediate in the presence of FAC

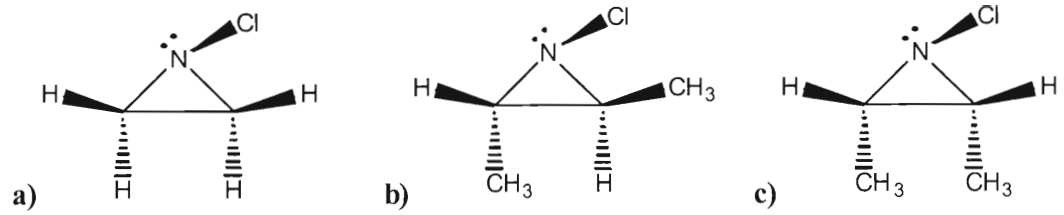


Figure I-10. 1-Chloroaziridine intermediates [36]: a) 1-chloroaziridine, b) 1-chloro-*trans*-dimethylaziridine, c) 1-chloro-*cis*-dimethylaziridine

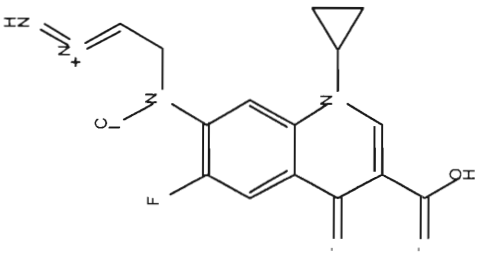


Figure I-11. Possible diimine structure for m/z 352

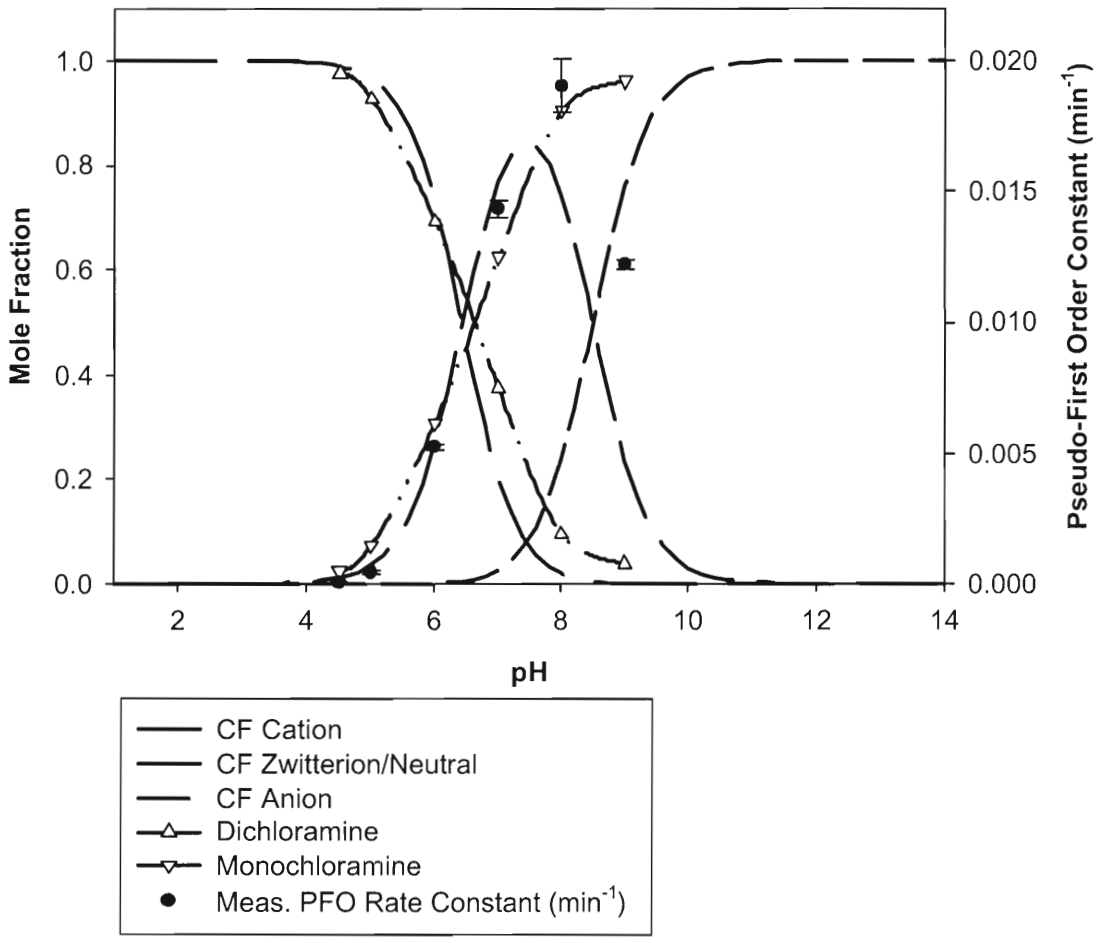


Figure I-12. pH-dependency of pseudo-first order rate constant for decay of CF intermediate (m/z 366) in presence of CC.

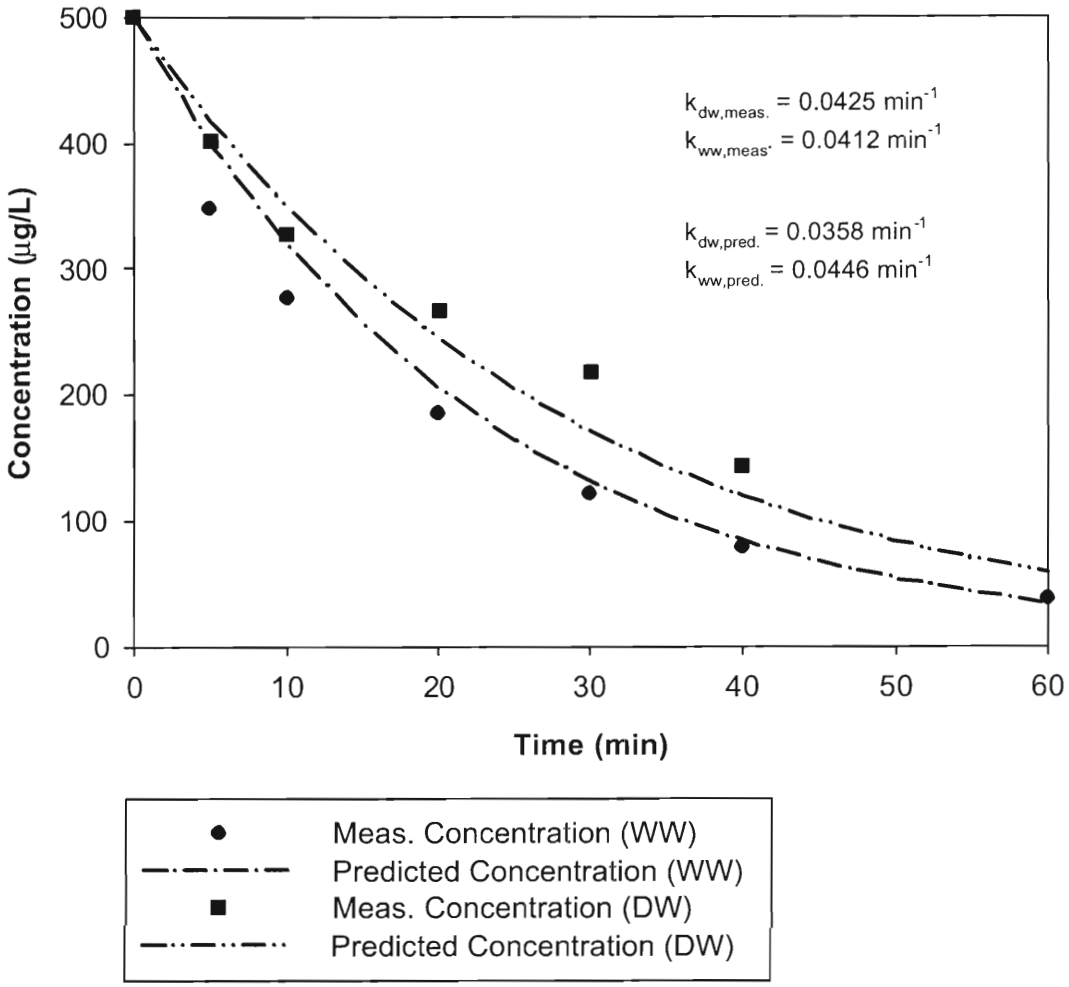


Figure I-13. CF Intermediate Decay Kinetics (pseudo-first order) in Real Water Systems

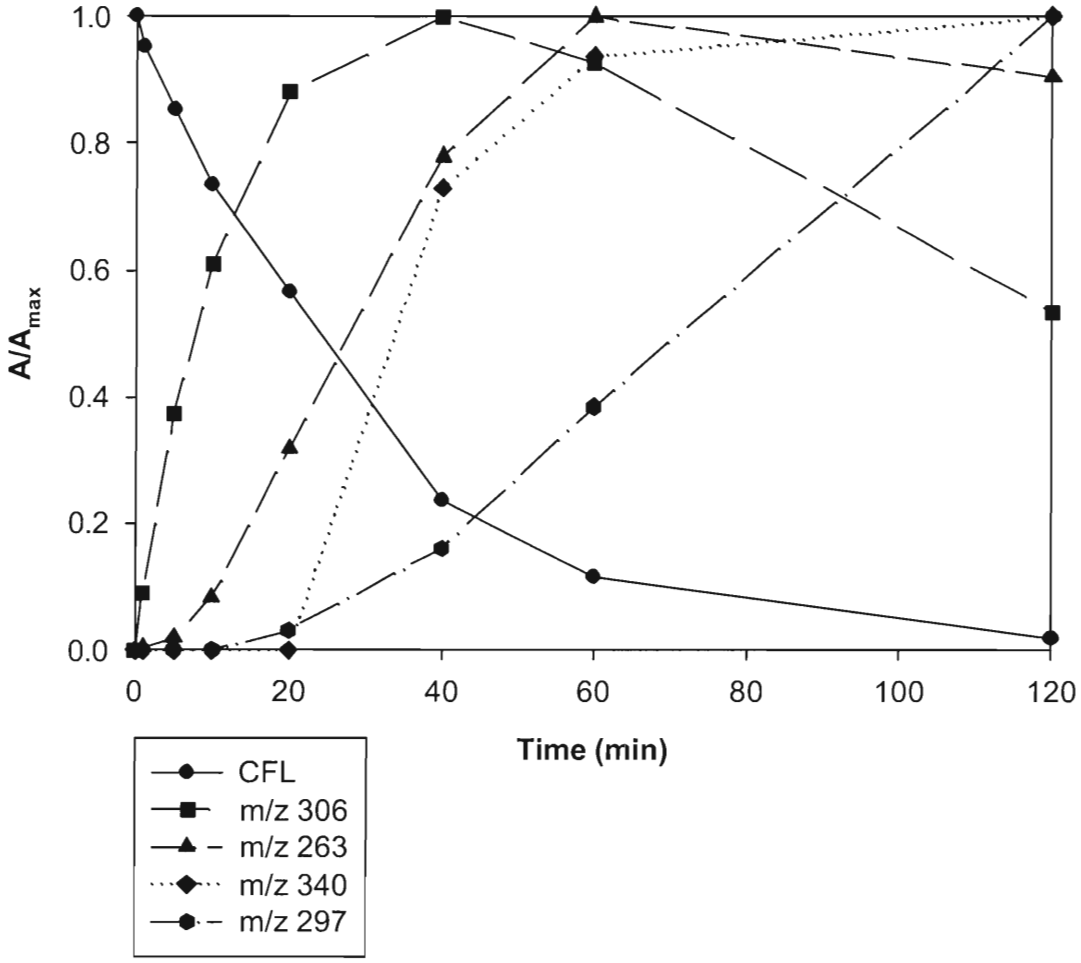


Figure I-14. Major oxidation product evolution for reaction of CF with FAC.

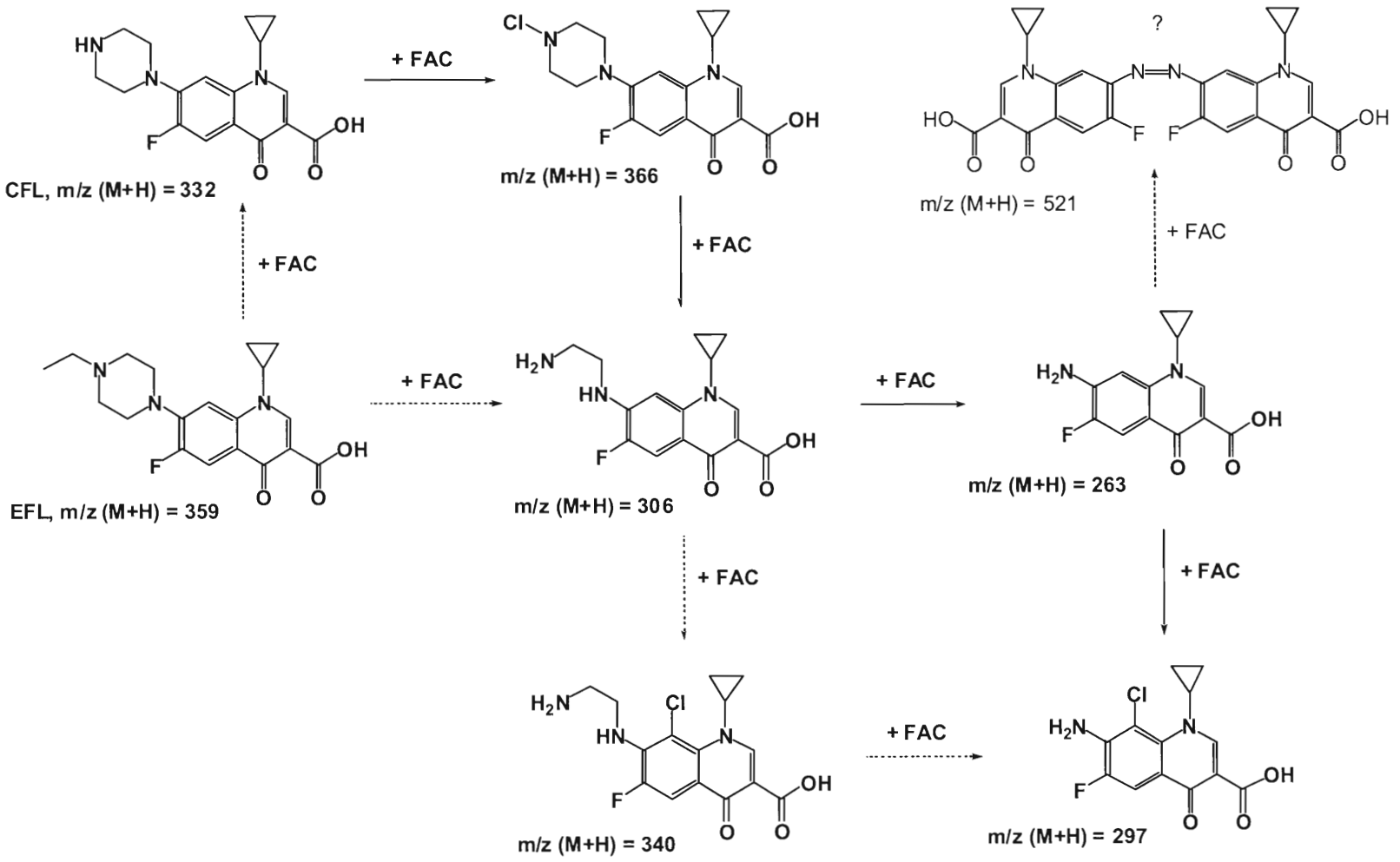


Figure I-15. Proposed degradation pathways for reaction of CF with FAC and CC

CHAPTER II. CHEMICAL OXIDATION OF THE ANTIBACTERIAL AGENTS SULFAMETHOXAZOLE AND TRIMETHOPRIM BY FREE AND COMBINED CHLORINE.

Introduction

Fluoroquinolones, sulfonamides, and the dihydrofolate reductase (DHFR) inhibitors – together with tetracyclines and macrolides – represent several of the most commonly used classes of modern antibiotics. The antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) – a sulfonamide and DHFR inhibitor, respectively (Table II-1) – are commonly prescribed in tandem, as well as on their own, and have been detected in relatively high concentrations (i.e., >100 ng/L) within municipal wastewater effluents in virtually every relevant occurrence study conducted within the past five years. Prior studies indicate that aerobic bacterial biodegradation of sulfonamides and TMP is extremely limited [10, 42, 43], providing one indication as to why these compounds might show up in such high amounts within municipal wastewater effluents and surface water bodies. On the other hand, significant photolytic degradation (by combinations of direct UV photolysis and radical-mediated mechanisms) of SMX and TMP has been reported [44-46]. Additional studies indicate extensive and rapid transformation of SMX and TMP by application of sodium hypochlorite [47, 48] to buffered aqueous systems.

Accordingly, reported results of several ongoing or recently completed studies have indicated substantial modification of antibiotics from the sulfonamide and DHFR inhibitor

families via engineered chemical oxidation processes, through the application of ozone, AOPs (ozone/hydrogen peroxide), or chlorine [49, 50]. Such findings would generally be expected, as a consequence of the presence of primary amino groups – typically susceptible to oxidative attack – within all compounds from these two classes. Observations from a recently completed occurrence study verify these expectations, as various sulfonamides and TMP are nearly completely degraded through chlorine and ozone disinfection processes in several municipal wastewater treatment plants within the United States [9, 17]. Thus, aqueous chemical oxidation mechanisms would appear to be efficient means of modifying – in some cases mineralizing – antibiotics from the sulfonamide and DHFR inhibitor families.

In the case of TMP, the end result of oxidative transformation seems to a significant reduction of biological activity, namely with respect to antibacterial efficiency [51]. However, several groups have suggested the possible role of reactive oxidative metabolites of SMX (e.g., hydroxylamines, nitroso-derivatives) and TMP (iminoquinone methide) in adverse reactions to these drugs in medical application [45, 47, 48]. In order to assess the potential ecotoxicological or human health risks of these compounds, it is important to first understand the rates and degree to which they are modified by pertinent chemical mechanisms (e.g., oxidation processes). Accordingly, this study was undertaken with the intent of quantifying reaction kinetics and clarifying the reaction pathways involved in oxidative degradation of TMP and SMX (Table II-1) and associated structural classes by free available chlorine (FAC) and combined available chlorine (CC) under conditions associated with chlorine-based municipal wastewater and drinking water disinfection processes.

Each compound exhibits three different species across the pH range 1 to 14. Thus, solution pH was expected to strongly influence the reaction rates between the HOCl/OCl⁻ and the compounds studied. Consideration of predominant antibiotic species and corresponding reaction rates at specific pH values was intended to provide a preliminary determination of reactive sites within the antibiotics. Second-order rate equations incorporating pH-dependent speciation of both substrate and chlorine oxidant have been developed previously to evaluate observed pH-dependent kinetics [15, 25, 52, 53]. A similar model has been developed and modified here in order to explain observed pH-dependencies of the reaction kinetics pertinent to each of the antibiotic compounds and free chlorine. Structurally-related compounds (Table II-1) that correspond to either the hypothesized reactive or inactive portions of SMX and TMP were examined in order to verify the proposed site(s) of reaction. LC/MS was utilized to identify reaction products and to assess product evolution during each reaction time course. Product characterization and temporal distribution provided further insights into reaction pathways and mechanisms. Additionally, oxidation of TMP and SMX in real environmental matrices has been used to assess applicability of observations from this study.

Materials and Methods

Standards and Reagents. SMX was obtained from ICN Biomedicals, Inc. (Irvine, California; USA). Trimethoprim (TMP), 2,4-dichloro-5-methylpyrimidine (DCMP), 3,4,5-trimethoxytoluene (TMT), 3,5-dimethylisoxazole (DMI), 3-amino-5-methylisoxazole (AMI), and 4-aminophenyl methyl sulfone (APMS) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri; USA). 2,4-diamino-5-methylpyrimidine (DAMP) was purchased from Daniels

Fine Chemicals, Ltd. (Edmonton, Alberta, Canada). 4-methyl-N-(5-methyl-isoxazol-3-yl)-benzenesulfonamide (MMIB) was purchased from ASINEX, Ltd. (Moscow, Russia). All standards used for preparation of stock solutions were reagent grade and were used without further purification. All reagent solutions (e.g., buffers, stocks, oxidants, quenching agents) were prepared using Nanopure© purified water. 100 mg/L stock solutions (for use in kinetic experiments) of all compounds were prepared in 10% methanol. 1 g/L stock solutions (for use in LC/MS product characterization studies and H^1-NMR analyses) of sulfamethoxazole and trimethoprim were prepared in 50% methanol. Stock solutions were discarded after two months of storage at <5° C.

~7% sodium hypochlorite solutions (in water) were obtained from Fisher Scientific International, Inc. (Pittsburgh, PA; USA) and diluted to ~100 mg/L (free chlorine) stock concentrations for use in kinetic experiments. Free chlorine stocks were periodically standardized iodometrically [20]. N,N-diethyl-*p*-phenylenediamine (DPD) was used through either DPD colorimetry or DPD-FAS titrimetry to measure free chlorine residual concentrations after completion of kinetic experiments [20].

Pre-formed chloramine stocks were prepared at pH values of 4.5, 5, 6, 7, 8, 9 in 0.1 M acetate (pH = 5), phosphate (6 = pH = 8), and borate (pH 9) buffers, with modifications to the methods of Chapin [21]. 302 mg/L solutions of NH_4Cl and 200 mg/L solutions of FAC (prepared from diluted 7% FAC stock and standardized iodometrically) were combined at 25°C in 1:1 proportion (to produce ~100 mg/L CC solutions, at 2:1 NH_4Cl:FAC molar ratios) under completely-mixed conditions, and allowed to react for no less than four hours, in order to ensure completion of dichloramine formation at lower pH values. CC stocks were prepared

prior to each experiment, temporarily stored at <5°C, and used within 24 hours of generation. CC concentrations were standardized using DPD-FAS titrimetry, in order to provide information on the distribution of monochloramine and dichloramine within each stock solution.

Buffered Deionized Water Systems. Temperature was maintained at 25°C in all experiments, using a NESLAB RTE-111 (Thermo NESLAB – Portsmouth, NH; USA) recirculating water bath connected to an acrylic water tank. Reaction solutions were partially immersed in the water tank and stirred with Teflon-coated stir bars using a 15-position magnetic stir-plate (Vario-MAG – Daytona Beach, FL; USA). A mercury thermometer was used to verify the temperature setpoint within the water tank.

0.01 M acetate (pH 3, 4, 4.5, 5), phosphate (pH 6, 7, 8), and borate (pH 9) buffers were used to maintain pH in all experiments. Addition of substrate and oxidant to buffered reaction solutions did not alter initial solution pH by more than 0.01 units. pH values of reaction solutions were checked after completion of each kinetic experiment to verify that solution pH did not vary by more than 0.05 units from starting values. Reaction kinetics for each antibiotic compound were assessed for at least seven pH values in the range pH 3 to 9. Kinetics for each surrogate and substructure were assessed at pH 4, 7, and 9.

Reactions involving SMX and TMP were initiated by addition of 100 mg/L FAC or CC stock (in 10:1 oxidant to antibiotic molar ratio for SMX and TMP) to 500 µg/L solutions of the respective antibiotic compound in 20 mL of buffer solution, under completely-mixed conditions, at 25°C. FAC reactions were monitored by quenching 1-mL samples of each reaction solution at appropriate time intervals and analyzing by HPLC with UV detection. A novel "soft" quenching technique, using a combination of NH_4Cl, tris-hydroxymethyl aminomethane

(THAM), and CH_3COOH has been utilized here to perform kinetic investigations into the reaction of SMX and TMP with FAC. Reaction of SMX with free chlorine yields at least one N-chloro intermediate that can be easily reduced and converted back to the parent compound by strong reductants such as sodium thiosulfate (Figure II-1a-c), thus negating the value of any kinetic measurements obtained through the use of relatively harsh quenching agents. In addition, reaction of TMP with free chlorine yields unstable products that are destroyed or further transformed by addition of $Na_2S_2O_3$ (Figure II-1d-f).

Experiments showed that NH_4^+/NH_3 can out-compete SMX or TMP for free chlorine at pH ~ 8.3, and that in the presence of excess NH_4^+/NH_3, free chlorine will not react with either compound. Additional experiments have shown that reactions between combined chlorine and SMX or TMP are extremely slow ($t_{1/2} >> 1$ d) at pH < 5, and that chlorine transfer from the respective N-chloro intermediates to free NH_4^+/NH_3 is negligible at acidic pH during a suitable analytical time-frame, thus indicating the applicability of NH_4Cl as a quenching agent under appropriate conditions. pH of samples was adjusted by spiking autosampler vials with appropriate amounts of THAM before addition of sample. An amount of NH_4Cl calculated to be in two-fold molar excess of maximum expected residual free chlorine was also added to the autosampler vials before sample addition in order to quench any residual oxidant. After addition of 1-mL samples from reaction vessels to the autosampler vials, pH of the samples was readjusted to <5 with a pre-determined amount of acetic acid in order to retard the disproportionation of organic N-chloramine intermediates and to prevent oxidation of residual parent compound by inorganic chloramine species. Required volumes of THAM and acetic acid were determined experimentally before each experiment by first measuring the amount of

THAM (0.01, 0.1, or 1.0 M) required to raise the pH of a 20-mL volume of each buffer solution to pH 8.3, then by measuring the volumes of acetic acid (0.1 or 1.0 M) necessary to lower the pH of each solution to <5 (except for the case of pH 9 buffer, which was first adjusted to pH 8.3 with 1.0 M acetic acid, and then further adjusted to pH <5). It is important to note here that the pH of experimental samples not be adjusted to below 4.5, as formation of NCl_3 – which may oxidize SMX and TMP – could lead to sample deterioration prior to analysis. Samples treated in this manner could be preserved for approximately two hours with less than 5% deviation of sample character from that of the the initial sampling time, so with expedient analysis, samples could be analyzed by HPLC in order to follow the behavior of normally unstable reaction systems with adequate resolution of sample peaks (Figure II-1). The reaction between FAC and SMX was followed over a two-minute time window, while the reaction between FAC and TMP was followed over 60-minute time windows.

CC reactions were monitored by HPLC analysis without quenching, immediately after sampling. In all cases except those involving the reactions of CC with SMX or TMP, for which 48-hour durations were utilized, kinetic investigations proceeded beyond at least one half-life for the particular compound of interest.

FAC and CC residual concentrations were measured by DPD colorimetry or DPD-FAS titrimetry [20] at the conclusion of each kinetic experiment in order to ensure applicability of pseudo-first order conditions. Sample dilution has been taken into account in determining concentrations of all analytes monitored by HPLC.

Real Water Systems. Water samples taken from a regional wastewater reclamation facility (WRF) and drinking water treatment plant (WTP) were used to assess the applicability

of observed reaction kinetics and temporal product distribution to wastewater and drinking water disinfection, respectively, as well as to evaluate the accuracy of the pH-dependency kinetic model developed as part of this study. 2-L samples of secondary wastewater effluent and filter box effluent were collected from the WRF and WTP, respectively. Samples were transported to the laboratory in sealed amber borosilicate bottles with Teflon caps, without preservatives, and stored on ice. The sample from the WRF corresponds to the point in the plant's treatment train just prior to application of chlorine gas, while the sample from the WTP corresponds to the point in the plant's treatment train subsequent to pre-chlorination by chlorine dioxide (to reduce chlorine demand) and immediately prior to disinfection by chlorine gas. Residual chlorine measurements provided by the plants verified the absence of available chlorine (free or combined) in either sample. These two samples provided representative examples of low ammonia (Table II-2) process waters typically subjected to disinfection by application of chlorine oxidants (e.g., ClO_2, NaOCl, Cl_2).

20-mL reaction volumes of each water sample were spiked with 500 µg/L of each antibiotic and subjected to free chlorine concentrations approximating those utilized by the plant from which they were derived ($C_{FAC,\ WTP}$ = 2 mg/L, $C_{FAC,\ WRF}$ = 10 mg/L). At each plant, the respective process waters are dosed with enough free chlorine to meet the water's chlorine demand and leave a residual of ~ 1 mg/L. Each reaction was followed in the same manner as for the clean water systems described above, with modification of the monitoring period for TMP in wastewater only (two-minute monitoring window).

LC/MS Product Characterization Studies. 2 mL of each 1 g/L antibiotic stock was added to 18 mL of 0.01 M pH 7 phosphate buffer to achieve starting concentrations of 100

mg/L, except for the case of TMP, for which oxidation was also studied using pH 4 acetate buffer. Free chlorine was added to solution from ~7% sodium hypochlorite stock to initiate oxidation at 2:1 molar ratios of FAC:antibiotic for SMX and TMP. 1-mL samples of each reaction mixture were added unquenched to autosampler vials for immediate analysis by LC/MS.

HPLC and LC/MS peak matching. Each antibiotic was added to pH 7 phosphate buffer to achieve 100 mg/L starting concentration as above, and dosed with FAC accordingly. 500 μL volumes of unquenched reaction solutions were separated by HPLC, and appropriate fractions were collected (using an ISCO Foxy, Jr. fraction collector) for each major product peak identified in kinetic studies. Dilute fractions were subsequently concentrated to approximately 500 μL by evaporating under nitrogen gas at ~50°C and analyzed by LC/MS to verify their identity using their respective m/z values.

Analytical Methods. An Agilent 1100 series HPLC system equipped with a 5 μm particle-diameter, 4.6 mm x 250 mm Zorbax RX-C18 column, a UV/Vis diode-array detector (DAD) was used to monitor residual concentrations of parent compounds and signal areas of products generated during the course of each oxidation reaction. Depending on the time constraints of each study or on chromatographic behavior of respective analytes, gradient and isocratic methods were used with varying ratios of 0.04 M H_3PO_4 buffer to pure acetonitrile (except in the case of 2,4-diamino-5-methylpyrimidine, for which a 0.001 M trifluoroacetic acid buffer was required to achieve satisfactory retention within the HPLC column).

An Agilent 1100 series HPLC system equipped with a 4.1 μm particle-diameter, 2.1 mm x 150 mm Zorbax SB-C18 column, a UV/Vis DAD, and an 1100 series single-quadrupole mass

spectrometer was used in the characterization of reaction products. Gradient elutions, using varying proportions of 0.2% (v/v) formic acid to pure acetonitrile, were used to separate product mixtures prior to analysis by electrospray ionization (ESI) at each of two fragmentation voltage settings (80 V and 120 V, for low and high degrees of fragment generation, respectively).

All GC/MS data were obtained using an Agilent 6890 gas chromatograph (w/ HP5-MS column) interfaced to an Agilent 5973 single-quadrupole mass spectrometer, in electron-impact mode (70 eV). . TOC analyses were conducted using a Shimadzu TOC-5050a Total Organic Carbon Analyzer and obtained according to standard methods for determination of non-purgeable organic carbon (NPOC) [20]. 1-dimensional H^1-NMR spectra, as well as 2-D COSY spectra, were obtained using a Bruker AMX 400 NMR instrument

H^1-NMR sample preparation. H^1-NMR was utilized to provide structural information in the absence of sufficient mass spectral data for an unknown oxidation product of SMX (described below, in Results and Discussion). 100 mL of a 200 mg/L SMX solution (unbuffered) was dosed with 168 mg/L FAC (to obtain a 3:1 oxidant:substrate molar ratio) and allowed to react for approximately 10 minutes, at which point a solid-phase extraction was begun by passing the reaction solution through a 5 mL Waters Oasis HLB SPE cartridge at ~1 mL/minute under vacuum. After extraction, a fraction containing relatively polar products was eluted from the SPE cartridge using 5 mL of a 75% methanol solution, followed by elution of the fraction of interest (Fraction 2) with 90% methanol, then by an elution of more hydrophobic products (primarily dimmers) with pure methanol. Fractions were recovered in 10-mL borosilicate glass test tubes. A 100 µL sample of Fraction 2 was analyzed by HPLC to verify

purity of the oxidation product (by assessment of DAD signal areas at 205 nm and 275 nm). The remainder of Fraction 2 was evaporated at room temperature under a gentle stream of N_2 gas until only an amorphous brown precipitate remained. This precipitate was subsequently dissolved in 0.5 mL of d_6-acetone and transferred to a 5-mm NMR tube for analysis by H^1-NMR.

Results and Discussion

Kinetic Studies in Clean Systems. Pseudo-first order kinetic rate constants were determined for SMX and TMP at various pH values in order to assess the effect of pH on reaction rate, and to allow a preliminary assessment of probable reaction sites for the attack of HOCl/OCl⁻ on each compound. Measured values of $\ln(C/C_0)$ were plotted against time (in minutes) in order to obtain the pseudo-first order rate constants (slope = $-k_{app}'$) in units of min^{-1}. An example plot is given in Figure II-2, for the degradation of SMX at pH=5. Linearity of pseudo-first order plots was checked via calculation of linearity coefficients, r^2, which ranged from 0.96 to 1. Each kinetic experiment involving SMX or TMP was conducted in triplicate, while those involving surrogate compounds were conducted in duplicate.. 95% confidence limits have been calculated and reported with all rate constants as error bars in relevant graphs.

Second-order rate constants were calculated according to procedures used by von Gunten and Oliveras [25]. Because oxidants (FAC or CC) were utilized in significant excess of substrates (10:1 oxidant:substrate on a molar basis), their total concentration $[FAC]_T$ or $[CC]_T$

can be assumed as constant over the course of reaction studied during this investigation. Thus, division of the measured pseudo-first order constant:

$$k_{app}' = k_{app}''[Oxidant]_T \text{, in units of } s^{-1}$$

by total oxidant concentration yields the apparent second order kinetic rate constant:

$$k_{app}'' = \frac{k_{app}'}{[Oxidant]_T} \text{, in units of } M^{-1}s^{-1}$$

Figure II-3 summarizes the results of pH-dependency studies for each of the antibiotics, plotted with speciation patterns of both the antibiotic and hypochlorous acid. A kinetic model has been developed to help explain variations in rate constants for degradation of antibiotic by FAC at the pH values studied, using a modified second-order elementary rate expression (1) for a bi-molecular system:

$$Oxidant + Substrate \rightarrow \Pr oduct(s); \quad \frac{\partial C_{Substrate}}{\partial t} = -k_{app}'' C_{Oxidant} C_{Substrate} \qquad (1)$$

Where k_{app}'' represents the second order rate constant for a particular reaction, $C_{Substrate}$ represents the total antibiotic concentration, and $C_{Oxidant}$ represents the total concentration of FAC (hypochlorous acid) in this case. However, this equation does not account for either substrate or oxidant speciation. In actuality $C_{Oxidant}$ and $C_{Substrate}$ are both comprised of contributions from various species. SMX and TMP both exhibit three primary species over the pH range 1 to 14 (Figure II-4), and the oxidant hypochlorous acid – for which $pK_a = 7.4$ [54] – exhibits two species (HOCl and OCl^-) within the same pH range. For a diprotic acid, species distribution can be represented by Eq. 2, where the compound's acid-dissociation constants are

K_{a1} and K_{a2} and the compound's species distribution coefficients are represented by α_1, α_2, and α_3:

$$C_{Substrate} = \alpha_0 C_{Substrate} + \alpha_1 C_{Substrate} + \alpha_2 C_{Substrate}, \quad (2)$$

$$\alpha_0 = \frac{[H^+]^2}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}}, \text{ for Species 0 (cation for SMX, diprotonated for TMP)}$$

$$\alpha_1 = \frac{K_{a1}[H^+]}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}}, \text{ for Species 1 (neutral for SMX, monoprotonated for TMP)}$$

$$\alpha_2 = \frac{K_{a1}K_{a2}}{[H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}}, \text{ for Species 2 (anion for SMX, neutral for TMP)}$$

Six individual reactions (comprised of the six possible combinations amongst the three antibiotic and two oxidant species) may be considered as contributing to the overall reactions between free chlorine and the antibiotic compounds. These six sub-reactions can be incorporated into the overall rate expression as shown in Eq. 3.

$$\frac{\partial C_{Substrate}}{\partial t} = -k_1[HOCl][Sp.0] - k_2[OCl^-][Sp.0] - k_3[HOCl][Sp.1] - k_4[OCl^-][Sp.1] - k_5[HOCl][Sp.2] - k_6[OCl^-][Sp.2] \quad (3)$$

where $k_{1\text{-}6}$ represent the specific second order rate constants corresponding to each combination of reactant species, and Sp. 0, 1, and 2 correspond to Species 1, 2, and 3.

Referring to the distribution coefficients for the substrate (antibiotic) as: α_0, α_1, and α_2; and to those for the oxidant (hypochlorous acid) as: α_0' and α_1' (for HOCl and OCl, respectively), Eq. 3 can be modified to yield Eq. 4. where [Ox.] and [Sub.] represent total concentrations of oxidant and substrate (all species), respectively.

$$\frac{\partial C_{Substrate}}{\partial t} = -k_1\alpha_0'[Ox.]\alpha_0[Sub] - k_2\alpha_1'[Ox.]\alpha_0[Sub] - k_3\alpha_0'[Ox.]\alpha_1[Sub] - k_4\alpha_1'[Ox.]\alpha_1[Sub] - k_5\alpha_0'[Ox.]\alpha_2[Sub] - k_6\alpha_1'[Ox.]\alpha_2[Sub] \quad (4)$$

Combination of this general model with experimental observations for each antibiotic compound permits calibration of the model to each compound's reactive characteristics. The general rate model can be further simplified by applying principal component analysis (PCA) [55] to determine which combinations of reactant species correspond to dominant sub-reactions in the overall reaction of substrate with oxidant. Application of Eq. 4 over a range of pH values yields a system of linear equations that is well suited to analysis via linear algebra (i.e., matrix mathematics). PCA can be applied to a matrix of $a_n a_m$' products (that is, the product of oxidant and substrate distribution coefficients) obtained for each pH value studied in a particular set of experiments to yield corresponding loadings of each sub-reaction with respect to identified principal components. This process can be used to determine which combinations of species will be present in greatest magnitude at a particular pH, and can in turn provide a preliminary estimate of which combinations of species (and hence, which sub-reactions within the set of six) are most important in determining the overall apparent second order rate constant.

The apparent rate constants ($k_{1,2}$ for SMX and $k_{1\text{-}3,6}$ for TMP) for reaction between hypochlorous acid/hypochlorite and the individual substrate species are calculated from experimental data using a matrix algebra routine written in MATLAB (see Appendix C), and the results are listed in Table II-3. The calculated rate constants suggest that reactions between HOCl and the cationic and neutral species of SMX significantly outweigh the other sub-reactions. Thus, the reaction involving oxidation of SMX by FAC can represented by Eq. 6:

$$\frac{\partial C_{SMX}}{\partial t} = -k_1\alpha_0'[Ox.]\alpha_0[Sub.] - k_3\alpha_0'[Ox.]\alpha_1[Sub.] \quad (6)$$

Similarly, the measured apparent rate constants suggest that reactions between HOCl and each of the three species of TMP, in conjunction with the reaction between OCl⁻ and neutral TMP, dominate the apparent reactivity. Principal component analysis indicates that Sub-reaction 1 (HOCl/diprotonated TMP) is less important than Sub-reaction 5 (OCl⁻/monoprotonated TMP), on the basis of relative concentrations of each reactant. However, a review of experimental observations indicates that this is not the case, as the reaction involving HOCl and the diprotonated species of TMP is actually quite important. Since the reaction between OCl⁻ and monoprotonated TMP is actually relatively minor (even as indicated by principal component analysis), this has been neglected, and Sub-reaction 1 has been used in developing the equation for TMP reactivity with FAC, instead. In turn, the reaction involving oxidation of TMP by FAC can be represented by Eq. 7:

$$\frac{\partial C_{TMP}}{\partial t} = -k_1\alpha_0'[Ox]\alpha_0[Sub] - k_3\alpha_0'[Ox]\alpha_1[Sub] - k_5\alpha_0'[Ox]\alpha_2[Sub] - k_6\alpha_1'[Ox]\alpha_2[Sub] \quad (7)$$

Using the simplified models, the overall apparent second order rate constant, k_{app}'', for oxidation of SMX or TMP by free chlorine in clean systems can be determined for any pH value within the range considered. Model fits based on these simplified models are also shown in Figure II-3a&b. As Figure II-3a&b indicate, the model fits align quite closely with the experimental data, indicating the success of the modified second order kinetic models in describing pH-dependencies of the reactions taking place within the experimental systems utilized in this investigation.

As the preceding discussion indicates, this pH-dependency model serves as a qualitative tool to evaluate the importance of each antibiotic and oxidant species in the overall oxidation

reactions by comparing the magnitudes of apparent constants corresponding to each sub-reaction. Such an approach aids identification of the sites of free chlorine attack on the antibiotics. As would be expected, experimental data suggest that attack of SMX and TMP by free chlorine is tied to protonation and deprotonation of basic nitrogen groups within each compound. The data suggest a direct correlation with protonation state of the amido-nitrogen (pKa_2) (i.e., the N within the sulfonyl amido linkage) in the case of sulfamethoxazole. While attack of the primary amino-nitrogen (i.e., the aniline nitrogen) group would certainly be reasonable, direct oxidative attack of the amido-nitrogen of SMX seems unlikely (this has been verified by use of a model compound, as will be discussed below). However, experimental and modeled pH profiles of kinetic rate variation do seem to correspond directly to the protonation/deprotonation of the sulfonyl amido nitrogen in SMX, as opposed to the aniline nitrogen's protonation/deprotonation patterns. Namely, reaction rates are highest where combinations of HOCl and the SMX anion are highest, and drop significantly with either protonation of the sulfonyl amido nitrogen or deprotonation of HOCl. An explanation for these observations will be discussed in detail below.

In the case of trimethoprim, the data suggest a direct correlation of reaction rate with protonation state of the heterocyclic nitrogen atoms – N_1 and N_3 – in the pyrimidine ring. This is also somewhat different than might be expected initially, as TMP's amino groups are potential sites of reactivity, but do not participate in protonation, except through resonance effects as shown in Figure II-5, where II and III represent resonance hybrids of I. Instead, direct protonation of trimethoprim occurs on the heterocyclic nitrogen atoms - N_1 and N_3 – in the pyrimidine ring, the former of which has been identified as the most basic site in the 2,4-

diaminopyrimidine structure (and to which pKa_2 applies) [56]. While counter-intuitive, this observation would seem to indicate that chlorine should attack the 2,4-diaminopyrimidine ring at the heterocyclic nitrogens, rather than the amino groups. Further support for this mechanism can be found in studies performed by Reynolds et al. [57] on the chlorination of the pyrimidine base cytosine, in which chlorine substitution on the N_1 and N_3 positions was indicated by H-NMR analyses of reaction products. Interestingly, trimethoprim exhibits an increase in reaction rate at pH < 5, indicating by inference that under acidic conditions, the compound's 3,4,5-trimethoxybenzyl group is readily oxidized by chlorine, as the N_1 group will be completely protonated, and the N_3 group increasingly protonated for pH = 5 (which should in turn result in decreasing susceptibility of the 2,4-diaminopyrimidine moiety to oxidative attack). Langvik et al. [58] have verified increased susceptibility of 3,4,5-trimethoxy-substituted aromatics to chlorine at acidic pH, while also noting relatively low reactivity of this moiety at pH > 5.

In order to verify the locations of proposed reaction sites, additional kinetic studies were performed in which substructures or structural surrogates of each antibiotic (Table II-1) were subjected to application of FAC. The reaction rate constants of APMS, DAMP, and TMT with chlorine are shown in Figure II-6. MMIB, DMI, and DCMP are unreactive with FAC (results not shown).

Evaluation of reactions involving SMX and its substructures leads to the following conclusions:

(1) Direct oxidative attack of the SMX molecule occurs on the amino-nitrogen, as indicated by the reactivity of APMS (which contains the aniline amino-nitrogen but no

sulfonyl amido-nitrogen) and non-reactivity of MMIB (which contains the sulfonyl amido-nitrogen, but not the aniline amino-nitrogen), respectively.

(2) The 3,5-dimethylisoxazole moiety of SMX does not participate in the oxidation by free chlorine, as indicated by the stability of DMI in reaction solution.

(3) Rate constants obtained from the chlorination of APMS compare very favorably in magnitude with those for chlorination of SMX, although pH-dependency of the rate constants exhibits a different pattern (influenced only by oxidant speciation, as opposed to antibiotic and oxidant speciation, since the pK_a for APMS is 1.83). This lends support to the conclusion that oxidation of SMX by FAC does involve attack on the amino-nitrogen. However, as noted in the pH-dependency of apparent kinetic rate constants for reaction of SMX with FAC, overall oxidation of the SMX structure is further modulated by protonation of the compound's amido nitrogen via an effect that is not completely clear at the current stage.

Oxidation studies involving TMP and its substructures indicate that:

(1) Reaction rates are influenced primarily by speciation of the 2,4-diaminopyrimidinyl moiety for pH = 5, while they are influenced by the interaction between the 3,4,5-trimethoxybenzyl moiety for pH = 5, as indicated by oxidation of DAMP and TMT. This indicates that oxidative attack of the TMP structure occurs primarily on the 3,4,5-trimethoxybenzyl ring under highly acidic conditions, and on the 2,4-diaminopyrimidine structure for mildly acidic to basic conditions. The 3,4,5-trimethoxytoluene moiety does not possess any functional groups capable of acting as

acids or bases, and thus does not exhibit pH-dependent speciation, indicating that the observed increase in its susceptibility to oxidative attack at low pH is likely either the result of acid catalysis or the influence of molecular Cl_2, though such causes have not been explicitly verified within the confines of the current investigation.

(2) Direct oxidative attack of the 2,4-diaminopyrimidine quite likely occurs on the heterocyclic nitrogens (predominantly N_1). This is supported by the relative basicity and proton affinity of the N_1 and N_3 atoms with respect to other N atoms within the 2,4-diaminopyrimdine structure [56, 59, 60]. In addition, other researchers' H-NMR analyses of chlorinated products from the reactions of free chlorine with the pyrimidine bases uracil and cytosine indicate the presence of chlorine atoms situated on nitrogens in the N1 and N3 positions for both bases [57, 61]. However, the possibility of direct attack on the exocyclic amino-nitrogens cannot be excluded on the basis of the current results. Reactions involving chlorination of a compound such as TMP could presumably proceed through a mixture of intermediate chloramines – heterocyclic and exocyclic alike. This possibility would seem to be corroborated by calculations performed for proton affinities of nitrogen centers in 2,4-diaminopyrimdine, which – while indicating maximum basicity in the N1 position – yield similar proton affinities for each of the amino nitrogen groups [59, 60]. In addition, a prior study has presented relatively similar theoretical electron charge densities for all four nitrogen atoms within the 2,4-diaminopyrimidine moiety [59], in turn indicating that the amino nitrogens (N_2 and N_4) could also represent attractive targets for electrophilic attack by an oxidant such as FAC.

DCMP stability in the presence of free chlorine should also be noted in this context, as its non-reactivity could be due to either substitution (i.e., replacement) of the N_2- and N_4-amino groups of 2,4-diaminopyrimidine by Cl, or the electron-withdrawing effect of these Cl substituents in lowering nucleophilicity of the heterocyclic N-atoms, or perhaps to a combination of both factors. Based on these kinetic analyses alone, it is impossible to discern here which oxidation mechanism (i.e., attack of heterocyclic or exocyclic nitrogens) predominates in the reaction of TMP with FAC.

Kinetic Studies in Clean Systems – CC. No attempt was made here to develop a predictive model similar to that utilized in FAC rate constant assessment, primarily on the basis of expected limitations on utility with regard to various systems. Chloramine equilibrium distributions (i.e., amongst NH_2Cl, $NHCl_2$, and NCl_3) are affected by a number of environmental parameters, and the general applicability of any model developed for a specific set of conditions would be extremely limited as a result of the difficulty in predicting expected changes in chloramine distributions with changes in system conditions. However, the results obtained here do provide valuable information regarding the relative reactivities of SMX and TMP with CC (namely, dichloramine and monochloramine), which is a common form of oxidant in municipal wastewater and drinking water disinfection processes.

Reactions between CC and SMX or TMP were extremely slow compared to those with FAC, as typified by a maximum measured second order rate constant of 4.2×10^{-1} $M^{-1}s^{-1}$ at pH 6 for SMX (Figure II-7), and 6.0×10^{-2} $M^{-1}s^{-1}$ at pH 7 for TMP (data not shown). In contrast, maximum measured reaction rate constants for reaction of SMX and TMP with FAC were 2.17×10^{3} $M^{-1}s^{-1}$ at pH 6 and 6.71×10^{1} $M^{-1}s^{-1}$ at pH 3, respectively. In fact, TMP exhibited

reactivity toward chloramines only at pH 7, after 39.5 hours of reaction time. This correlates relatively well with expected reactivities, based on consideration of oxidation strengths for each oxidant species relevant to the reactions studied here, where order of oxidant strength follows the trend $HOCl>OCl^->NHCl_2>NH_2Cl$ [39]. A review of the kinetic data for TMP and SMX oxidation by FAC indicates that reaction with OCl^- is much slower than with the protonated acid HOCl. Thus, reaction rates between $NHCl_2$ or NH_2Cl and the two antibiotics should be significantly slower than observed for FAC oxidation. The relative weakness of $NHCl_2$ as an oxidant, with respect to SMX, is also clear from Figure II-7, in which a significant decrease in reactivity is clearly evident in direct parallel with a decrease in the mole fraction of CC as dichloramine. Results for reaction of the antimicrobial agent ciprofloxacin (CF) with CC (discussed in Chapter 1) would seem to validate this explanation, as CF – which is oxidized readily by OCl^-, is also oxidized by both monochloramine and dichloramine [62].

An additional explanation for the observed low reactivity of chloramine oxidants for SMX and TMP could involve relative rates of chlorine transfer between N-chlorinated species (inorganic or organic) and inorganic or organic substrates. As described in an earlier study conducted by Yoon and Jensen [38], rates of chlorine transfer from representative substrates such as glycine, glycylglycine, and methylamine to free ammonia are appreciable, though approximately two orders of magnitude lower than for transfer in the opposite direction (i.e., from monochloramine to organic substrates). Using the example provided by methylamine, for which the $k_{m,o}$ (monochloramine to methylamine) and $k_{oc,a}$ (N-chlorinated methylamine to ammonia) rate constants have been measured as 0.323 and 5.85×10^{-3} $(mol/L)^{-1}s^{-1}$, respectively, a comparison of expected chlorine transfer rates can be made for the reaction

conditions employed in SMX and TMP studies. Under conditions of the current investigation, initial SMX and TMP concentrations are 1.97 μM and 1.72 μM, respectively. Each reaction solution was dosed with pre-formed CC (at a 2:1 molar NH_4^+:FAC ratio) to yield a 10:1 CC:substrate ratio. Accordingly, the concentration of oxidant present should be equal to or less than the concentration of unreacted ammonia in these reaction solutions (taking into account the 1:1 and 2:1 stoichiometry of FAC:$(NH_4^+)_T$ reactions at basic and acidic pH values, respectively). Therefore, [substrate] ~ 2×10^{-6} M and [CC] ~ $[NH_4^+]_T$ ~ 2×10^{-5} M represent the conditions of this study. Using methylamine as a model compound, under the conditions of this study, calculations should yield a predicted monochloramine to organic transfer rate of 1.29×10^{-11} M/s and an N-chlorinated substrate to ammonia transfer rate of 2.34×10^{-13} M/s (assuming the concentration of N-chlorinated organic equals 2×10^{-5} M, i.e., that all of the organic substrate is chlorinated). Experiments performed in the current study indicate chlorine transfer from N-chlorinated SMX or TMP products to free ammonia, as indicated by a slow (on the order of hours) decrease in N-chlorinated species and accompanying increase in parent compound, in the presence of excess ammonia. The phenomenon of parent substrate regeneration by chlorine transfer from the N-chlorinated substrate to free ammonia likely renders the reaction of SMX with CC to appear even slower than it actually is. Uetrecht et al. [47] noted a similar regeneration of parent substrate upon addition of reducing agents such as ascorbic acid or phenylbutazone to solutions containing N-chlorinated sulfamethoxazole.

Although the above calculations indicate a relatively large difference in forward and backward reaction rates of chlorine transfer (i.e., ~two orders of magnitude), and thus do not likely completely explain the apparent low reactivity of SMX and TMP in the presence CC,

consideration of one additional reaction pathway may help to account for this disparity. Monochloramine and dichloramine could be expected to react with excess free ammonia in preference to unreacted substrate, on the basis of our observations regarding the reactions between FAC and unreacted antibiotic species in the presence of excess NH_4Cl. Addition of FAC to buffered solutions containing a two-molar excess (relative to FAC) of NH_4Cl and 500 μg/L of either SMX or TMP did not result in any decrease of substrate concentration over the course of several hours, indicating that **(i)** all FAC reacted with free ammonia, instead of reacting with the antibiotic compounds, and **(ii)** the generated chloramines do not react with the antibiotics at appreciable rate. On the basis of redox chemistry, one would expect **(i)** to be due to greater electronegativity of the ammonia nitrogen relative to reactive sites present within the organic substrates, and thus higher reactivity of ammonia toward oxidant species such as chlorine. This should be analogous to reactions involving chloramines **(ii),** in which chloramine oxidant species could be expected to exhibit greater affinity for free ammonia than for free organic substrates.

Accordingly, the results obtained for reactions involving CC and TMP or SMX are likely indicative of a combination of effects due to relative oxidant species strengths, reverse chlorine transfer reactions (N-chlorinated substrates to ammonia), and competition between free ammonia and unreacted organic substrate for oxidizing chlorinated species. Results obtained here have served as a foundation for the novel chlorine-quenching technique described in the Materials and Methods section of this chapter.

Kinetic Studies in Real Water Systems. Kinetic experiments were conducted in water samples obtained from a WRF and a WTP in order to determine the degree of accuracy with

which kinetic calculations based on model predictions could be applied to actual water treatment systems. Reactions were studied in both wastewater and drinking water process samples to gauge the relative effect of competing chlorine scavengers and other complicating factors in systems for which chlorine disinfection is most likely to be used in current water treatment practice. Observed oxidation kinetics for each antibiotic corresponded to those predicted by the second order model relatively closely, where the model generally under-estimated oxidation rates for SMX, and over-estimated oxidation rates for TMP. The reason for these deviations is not clear at this time. SMX could potentially be out-competed for FAC by chlorine scavengers present in these water matrices. On the other hand, the influence of some form of catalyst present in wastewater samples studied here with regard to oxidation of TMP by FAC could be one possible explanation for the differences in TMP reactivity within wastewater as compared to clean water systems.

Additionally, the rates at which each antibiotic was oxidized in the real water samples indicate that complete conversion of the parent compounds could also be expected for residence times typical of wastewater disinfection chambers (10-30 minutes) and drinking water clearwells (1-24 hours). In light of the relatively high concentrations of antibiotics utilized for kinetic studies in this investigation, however, additional experiments utilizing more realistic in situ concentrations could provide a more certain conclusion in this regard. On the other hand, support for these experimental observations can be provided at full-scale by comparing to results obtained from a recently completed occurrence survey of antibiotic compounds in municipal wastewaters of the United States [9, 17], which showed substantial decreases in aqueous concentrations of SMX and TMP after application of chlorine disinfection.

As will be discussed below, reaction product distributions associated with oxidation of the antibiotics in wastewater or drinking water matrices correlate well with those previously obtained for D.I. water systems.

LC/MS Product Characterization. LC/MS analysis of reaction solutions for each antibiotic compound indicated a wide array of both unstable intermediates and stable end-products:

SMX: Mass chromatograms corresponding to oxidation products of SMX yielded a number of distinct, stable peaks (Table II-4). Two transient peaks, both corresponding to m/z (M+H) 288 appear to be an N-chloro intermediate and a transient ring-chlorinated SMX molecule. A prominent, early-eluting peak in *HPLC and LC/MS chromatograms* ($?_{max}$ = *210 nm, m/z* (M+H) = 99) was identified as 3-amino-5-methylisoxazole (AMI). The identity of AMI was confirmed by comparison of spectral characteristics and corresponding retention times to a pure standard.

A very prominent peak (Product 5, i.e, Peak 5 in Table B4) detected by way of its UV absorption (abs. maximum at 295 nm) in both kinetic experiments and product characterization studies involving the reaction of SMX with FAC failed to ionize in either APCI or ESI modes in LC/MS analyses. Experimental data seem to suggest that this is a major product of the reaction – it is present throughout the duration of kinetic studies, and yields the highest signal of any product peak in DAD chromatograms obtained from SMX kinetic and product characterization studies. The peak was stable over the course of at least 48 hours in the absence of chlorine or reducing agents. Addition of sodium thiosulfate or ascorbic acid rapidly depleted the peak, yielding various polar products (as indicated by increased peak abundance for early elution

times on HPLC and LC/MS). Isolation of Product 5 by collection of appropriate HPLC fraction and subsequent treatment with potassium iodide indicates that it possesses at least one active chlorine, as oxidation of the iodide to iodine immediately yields a yellow color.

Variation of buffer type (water, 0.2% acetic acid, and 0.2% formic acid were all tested), spray chamber temperature, drying gas temperature, drying gas flow rate, and fragmentation voltage did not seem to influence ionization efficiency of Product 5 in ESI or APCI-MS. The unknown apparently survived separation through gas chromatography, as it was detected by electron impact MS. However, the mass spectrum obtained from this peak yielded only fragments corresponding to low mass ($m/z<141$) singly-chlorinated ion peaks, indicating excessive fragmentation through this technique. The "softer" technique of chemical ionization MS could possibly reduce the problem of excessive fragmentation, but was not available at the time of these experiments.

H^1-NMR was utilized to provide structural information in the absence of mass spectral data. The NMR spectrum for pure SMX (Figure II-9) yields peaks at chemical shifts d=2.1 (singlet, 3 H), d=6.1 (singlet, 2 H), d=6.4 (singlet, 1 H), d=6.8 (doublet, 2 H), d=7.8 (doublet, 2 H), and d=9.8 (singlet, 1 H). Analysis of Product 5 yields a spectrum containing relevant peaks at d=6.4 (doublet, 1 H), d=6.8 (doublet, 1 H), d=7.6 (singlet, 1 H), and d=8.1 (doublet, 1 H). Signals corresponding to the methyl group and heterocyclic proton of SMX's isoxazole ring are conspicuously absent from the Product 5 spectrum (Figure II-9).

These NMR spectra indicate that the sulfonyl amido nitrogen somehow participates in the chlorination reaction – resulting in cleavage of the SMX structure at this particular site. Analysis of samples obtained from kinetic experiments conducted at pH 6.5 further verifies that a peak

corresponding to that of pure AMI appears at roughly the same reaction time as Product 5, and persists throughout the reaction monitoring period. The relative prominence of AMI and the Product 5 in HPLC and LC/MS chromatograms would also seem to indicate a 1:1 correspondence between the two – leading to the conclusion that the two products (AMI and Product 5) are generated in equal proportion from cleavage of the parent SMX, and that the unknown most likely corresponds to a modified sulfanilic acid moiety. UV spectra of Product 5 indicate that the aromatic structure of the benzene ring has been disrupted in some way (Figure II-10). Similarly, the changes in shifts of the four aromatic protons detected in H^1-NMR spectra, also suggest substitution or alteration on the aromatic ring. The pronounced increase in absorbance of the Product 5 (relative to SMX) at 295 nm – corresponding to observed absorbance maxima for inorganic and organic dichloramines [63], coupled with the positive test for oxidizing potential, indicate that Product 5 could be an N,N-dichloro-substituted variant of sulfanilic acid, which might explain (in part) the instability of the molecule during MS analyses. Additionally, a prominent shoulder in the UV spectrum for Product 7 (Peak 7 in Table II-4) correlates with absorbance at 254 nm, which corresponds to the maximum absorbance wavelength for inorganic and organic monochloramines. This provides support for identification of Product 7 as an N-chloramine intermediate, as opposed to a stable ortho-substituted chlorinated sulfamethoxazole.

TMP: Reaction mixtures obtained from the oxidation of TMP by FAC yield considerably more complex chromatograms than reactions involving SMX. Numerous stable substitution products were identified (Tables II-5 and II-6). Interestingly, a small peak corresponding to one stable product was identified as containing four Cl atoms (molecular ion of m/z 445, and three

isotopic peaks at 447, 449, and 451), indicating that substitution can occur in numerous locations on the TMP structure. Fragmentation of this compound was minimal. Unstable products which appeared to be intermediates (likely N-chlorinated) exhibited mass spectra corresponding to: m/z 377 (isotopic peak at m/z 379 ? 2 Cl) and m/z 411 (isotopic peak at m/z 413 ? 3 Cl). The fragment ion peak of m/z=181 in TMP product mass spectra apparently corresponds to the 3,4,5-trimethoxytoluene fragment of the molecule, cleaved from the parent ion at the aliphatic carbon bridging it to the 2,4-diaminopyrimidine moiety.

Proposed Reaction Pathways – Reactions with FAC.

SMX: A review of the literature on the application of protecting groups in synthetic chemistry highlights the use of tosyl groups (typically introduced by use of *p*-toluene-sulfonyl chloride) to protect relatively delicate amino nitrogens during synthesis reactions by converting them to sulfonamide nitrogens. The tosylation process creates a highly stable S-N bond, which is by its intended nature extremely difficult to cleave. A resurgence in interest related to gentle deprotection of the amino group (by cleavage of the S-N bond) has resulted in the development of a number of techniques involving metallic reductants, acid-catalyzed hydrolytic reactions, and photolytic bond cleavage. Of these, the photolytic pathway seems to bear the closest relation to mechanisms likely to be involved in the FAC/SMX reaction system.

Direct photolysis of sulfonamide groups at near-UV wavelengths below 300 nm can result in unintended destruction of side-chain chromophores. In turn, several groups have pursued development of electron-transfer radical chains utilizing sacrificial absorbers such as 1,4-dimethoxybenzene and 1,5-dimethoxynaphthalene or the ß-naphtoxide ion to initiate radicalization of the sulfur center in sulfonamide groups, which has been shown to lead to

subsequent cleavage of the S-N bond and relatively high recoveries of starting amines. Though not explicitly described in their work, a similar system would appear to have been at work in a study conducted by Zhou and Moore [45] on photolysis of SMX. Analogy between the photolytic pathway and the chlorination reaction can be found in consideration of the mechanisms by which N-chlorinated anilines have been shown to decay. Three pathways have been previously described to explain the means by which N-chloroanilines rearrange or decay: 1) passage through a cationic anilinium state, by Cl-N cleavage to yield Cl^- and positively-charged N [64], 2) transition through an anionic amide state, characterized by Cl-N cleavage to yield Cl^+ and negatively-charged N [64], and 3) transition through the aminium radical [37]. Action of the aminium radical would seem to provide a reasonable explanation for observed activity in the case of the reaction of FAC and SMX, as radicalization of the sulfonamide S-N bond could foreseeably proceed through an electron-transfer directly from an excited aminium radical, leading to the observed S-N bond cleavage. The fact that MMIB – for which the activating amino group is absent – is completely inert in the presence of even extreme excesses of FAC also provides support for the conclusion that radicalization of the *p*-amino group of the aniline moiety is required to initiate radical-chain propagated cleavage of the S-N bond, and also indicates that the sulfonamide group is not susceptible to direct electrophilic attack by FAC. Thus, the reaction between FAC and SMX can be envisioned as one in which initial attack of the SMX structure occurs at the aniline-nitrogen, followed by subsequent non-specific attack of <u>any</u> sulfonamide S-N bond remaining in the reaction system. The parallel nature of the chloramination and radical S-N cleavage mechanisms could also provide an explanation for the observed pH-dependency of SMX oxidation by FAC. Support for generation of aminium radicals in the absence of metal or photolytic chain initiators (i.e., under mild conditions) can be

found in the extensive research conducted on oxidation of amines (via hypochlorous acid and chlorine dioxide) by the Research Laboratories of the U.S. Army Edgewood Arsenal [26-28]. Figure II-11 portrays the evolution of major products identified through peak matching between data obtained via HPLC and that obtained in LC/MS studies for SMX. SMX/CC product distributions are very similar to those observed for SMX/FAC. Proposed degradation pathways for the reactions of SMX with FAC or CC are illustrated in Figure II-12.

TMP: The fragment ion of m/z 181 (described above) only appears in mass spectra (even at 120 eV fragmentation voltage) after treatment of TMP with FAC. The parent TMP does not yield such a fragment upon ionization, indicating that chlorine somehow sensitizes TMP to fragmentation at the aliphatic carbon. Lai et al. [48] have proposed the formation of a highly reactive iminoquinone methide molecule during chlorination of TMP, in which the antibiotic's aliphatic carbon participates in resonance with the aromatic heterocyclic ring of the 2,4-diaminopyrimidine (Figure II-13). This would in turn make the aliphatic (now olefinic) carbon a much more attractive site for oxidative attack, and could provide an explanation as to the mechanism by which TMP is sensitized to fragmentation in the reaction with chlorine. Mono- or di-substitution of chlorine on such an olefinic carbon could be expected to result in decreased stability of its bonds to neighboring carbons under conditions characteristic of ESI/MS. Correspondingly, a chlorinated fragment (e.g., m/z 215) resulting from breakage of the bond at the olefinic carbon position during ionization would contain chlorine at a terminal carbanion position, and would likely be unstable in itself. Yields of m/z 181 (- 1Cl) would be expected to be relatively high in this case. Thus, m/z 181 (or any other trimethoxytoluene fragment, for that matter) appear to be indicators of chlorination at the aliphatic carbon position in TMP. N-

acetyl-L-cysteine was used to trap the iminoquinone methide and allow subsequent characterization in the study undertaken by Lai et al., so its absence in mass spectra obtained for reaction mixtures in the current investigation is not surprising.

The character ascribed to the trimethoxytoluene fragment (m/z 181) in ESI/MS has been verified by studying mass chromatograms of TMP/FAC reaction mixtures for pH 4. As described earlier, oxidation of the TMP structure appears to take place primarily on the trimethoxytoluene portion of the molecule for pH<5. In turn, one would expect mass spectra corresponding to resulting products to exhibit either an abundance or absence of stable chlorinated trimethoxytoluene fragments (m/z 215 or 249), and an absence or relative scarcity of trimethoxytoluene fragments (m/z 181), as chlorine substitution on the aromatic trimethoxy ring should yield very stable fragments. Such expectations were fulfilled, as m/z 215 was present for numerous chlorinated products in the pH 4 mixture, and m/z 249 was observed for three prominent, highly-chlorinated reaction products. m/z 181 was observed for only one low-abundance peak, even by high-voltage analyses. One additional conclusion that can be taken from these analyses (as indicated by the ubiquity of the m/z 215 fragment ion) is that the olefinic carbon of the transient iminoquinone methide structure is quite amenable to oxidative attack even at low pH, pointing toward participation of this proposed intermediate in reactions throughout the range of pH values studied. Projected reaction pathways have been developed utilizing a combination of all kinetic data, substructure studies, and product characterization analyses discussed to this point. Figure II-14 portrays the evolution of major products identified through peak matching between data obtained via HPLC and that obtained in LC/MS studies for each antibiotic. TMP did not exhibit any appreciable degradation in the presence of CC,

even after 39.5 hours. Proposed degradation pathways for the reaction of TMP with FAC are illustrated in Figure II-15.

Conclusions

SMX is oxidized quite rapidly by FAC (10 s < $t_{1/2}$ < 5 minutes) at pH values between 4 and 9, and appears to react most quickly at pH ~ 6.5, which falls within the range of pH values likely to be encountered in water treatment disinfection processes. As noted, its primary N-chlorinated intermediate can be reduced relatively easily upon reaction of a strong reducing agent to yield the parent compound, indicating that – at least under conditions of wastewater treatment, for which dechlorination is a relatively common practice after chlorine disinfection – a significant amount of chlorinated SMX could be regenerated prior to final discharge of wastewater effluent into the environment. Experiments conducted in real water systems verify predictions made using data obtained from clean systems. The relatively rapid oxidation of SMX is accompanied by what appears to be a unique radical-chain cleavage of the S-N sulfonamide bond to yield AMI and a modified sulfanilic acid moiety. S-N bond cleavage, combined with N-chlorination of the aniline functional group appears to lead to the formation of relatively stable dimers in addition to lower mass products.

TMP, on the other hand, undergoes relatively slow oxidative transformation compared to SMX (15 min. < $t_{1/2}$ < 60 min.) under the conditions utilized in this study for clean systems. However, owing to undetermined causes, TMP reacts extremely fast in the presence of significant amounts of excess FAC (i.e., > 10:1 oxidant:substrate molar ratio), as exhibited by its

fast degradation in samples obtained from the WRF, and so will likely still be substantially transformed under conditions of both wastewater and drinking water disinfection processes. In contrast to SMX, chlorination of TMP yields primarily stable, multiply-substituted products – at least within the timeframes evaluated in this study – otherwise undergoing relatively minor structural modification.

Table II-1. Selected Antibiotic Compounds and Associated Structures

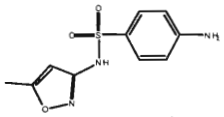
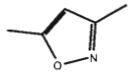
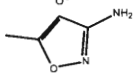
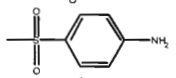
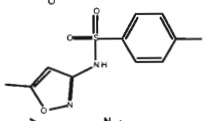
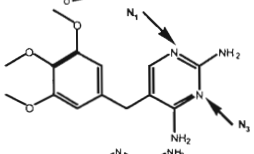
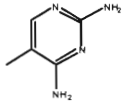
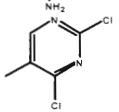
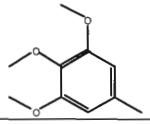
Compound	Structure	Molecular Weight	pK_a
sulfamethoxazole (SMX)		253.28	pKa1=1.83, pKa2=5.57 [65]
3,5-dimethylisoxazole (DMI)		97.12	NA
3-amino-5-methylisoxazole (AMI)		98.1	pKa_1=2.63 [66]
4-aminophenyl methyl sulfone (APMS)		171.22	pKa_1=1.48 [67]
4-methyl-N-(5-methyl-isoxazol-3-yl)-benzenesulfonamide (MMIB)		252.29	pKa_1=10.58 [66]
Trimethoprim (TMP)		290.32	pKa1=1.32 [68], pKa2=7.45 [69]
2,4-diamino-5-methyl pyrimidine (DAMP)		124.14	pKa_1=5.15, pKa_2=7.54 [66]
2,4-dichloro-5-methylpyrimidine (DCMP)		163.00	NA
3,4,5-trimethoxytoluene (TMT)		182.22	NA

Table II-2. Water quality data for wastewater and drinking water samples

Water Quality Data	pH[a]	Alkalinity (mg/L)[b]	NH_3 (mg NH_3-N/L)[c]	TOC (mg/L)
WRF[d]	7.33/7.30	120/127	<MDL/<MDL*	14.0/12.1[a]
WTP	6.60	13	<MDL*	1.25[b]

[a]Measured in laboratory, [b]Measured by plant personnel, [c]Measured by plant personnel and verified in laboratory, [d]First measure for wastewater sample obtained on 2/11/03, second for 2/13/03, *MDL=0.12 mg NH_3-N/L

Table II-3. Principal reactions for oxidation of SMX and TMP by FAC

Compound	Principal Reaction	Calc. Specific Second Order Constant ($M^{-1}s^{-1}$)
SMX	HOCl + SMX Cation	1070
	HOCl + SMX Neutral	2420
	OCl^- + SMX Anion	~ 0
TMP	HOCl + TMP Diprotonated	2900
	HOCl + TMP Monoprotonated	7.9
	HOCl + TMP Neutral	237
	OCl^- + TMP Neutral	5.4

Table II-4. Oxidation products of reaction between FAC and SMX, as analyzed using LC/MS (80 eV fragmentation voltage unless noted otherwise).

#	RT (min)	$M+H^+$	Compound	m/z Fragments
1	7.704	99	AMI	**99**, 72 (18%)
2	8.604	190	Sulfanilic acid + OH	**190**, 148(5%), 147 (5%)
3	11.46	193	AMI Dimer	**193**, 161 (9%), 99 (5%)
4	12.418	254	SMX	**254**, 156 (13%)
5	14.17[a]	?	Major product	?
6	15.833	288	o-chloro-SMX	**288** (290, 292), 254 (16%), 117 (80%)
7	16.713	288	N-chloro-SMX	**288** (290, 292), 253 (44%), 190 (92%, 192), 127 (23%)
8	20.279	501	Dimer	**501**, 437 (62%), 410 (96%)
9	21.193	503	SMX Dimer	**503**, 439 (6%), 412 (7%)
11	23.378	377	Dimer	**377** (379, 380)

[a]Product 5 yielded no mass spectrum, RT provided is for analyses by Agilent 1100 UV/Vis module.

Table II-5. Oxidation products of reaction between FAC and TMP, as analyzed using LC/MS (80 eV fragmentation voltage unless noted otherwise) - pH 7.

#	RT (min)	$M+H^+$	Compound	m/z Fragments
1	9.327	359	Sub. TMP	**359** (361, 363), 181 (17%)
2	9.685	291	TMP	**291**
3	10.3	373	Sub. TMP	373 (76%, 375, 377), **291**, 181 (36%)
4	11.134	325	Sub. TMP	**325** (327, 329), 291 (9%)
5	12.356	377	Sub. TMP	**377** (379, 381), 343 (5%), 181 (22%)
6	14.945	377	Sub. TMP	**377** (379, 381), 181 (51%)
7	15.804	391	Sub. TMP	**391** (393, 395), 357 (5%, 359), 181 (19%)
8	16.491	393	Sub. TMP	**393** (395, 397), 357 (5%, 359), 321 (8%), 215 (29%, 217), 181 (41%)
9	17.752	411	Sub. TMP	**411** (413, 415, 417), 377 (21%, 379, 381), 291 (39%), 181 (40%)
10	18.122	377	Sub. TMP	**377** (379, 381), 181 (32%)
11	19.154	407	Sub. TMP	**407** (409, 411), 377 (9%, 379), 215 (48%, 217), 181 (30%)
12	20.631	411	Sub. TMP	**411** (413, 415, 417), 375 (24%, 377, 379), 339 (16%, 341), 215 (11%, 217), 181 (29%)
13	23.215	445	Sub. TMP	**445** (447, 449, 451, 453), 427 (15%, 429)
14	24.893	425	Sub. TMP	**425** (427, 429, 431), 391 (20%, 393), 357 (15%, 359), 181 (56%)

Table II-6. Oxidation products of reaction between FAC and TMP, as analyzed using LC/MS (80 eV fragmentation voltage unless noted otherwise) - pH 4.

#	RT (min)	$M+H^+$	Compound	m/z Fragments
15	11.854	291	TMP	291
16	14.399	377	Sub. TMP	**377** (379, 381), 215 (18%, 217)
17	15.742	325	Sub. TMP	**325** (327, 329)
18	17.073	359	Sub. TMP	**359** (361, 363), 325 (17%, 327)
19	17.446	411	Sub. TMP	411 (44%, 413, 415, 417), **359** (361, 363), 325 (26%, 327), 215 (13%, 217)
20	18.37	359	Sub. TMP	**359** (361, 363), 325 (18%, 327)
21	19.647	425	Sub. TMP	**425** (427, 429), 377 (39%, 379), 359 (53%, 361), 325 (47%), 215 (32%, 217), 181 (26%)
22	21.035	427	Sub. TMP	**427** (429, 431, 433), 249 (45%, 251, 253), 215 (56%, 217)
23	22.084	411	Sub. TMP	**411** (411, 413, 415, 417), 325 (12%), 215 (29%, 217)
24	24.328	445	Sub. TMP	**445** (447, 449, 451), 249 (22%, 251), 215 (38%, 13)
25	27.882	459	Sub. TMP	**459** (461, 463, 465), 249 (21%, 251), 215 (66%, 19)
26	28.594	445	Sub. TMP	**445** (447, 449, 451), 375 (17%, 377), 344 (19%), 249 (43%, 251), 215 (47%, 217)
27	28.955	393	Sub. TMP	**393** (395, 397, 399), 323 (7%, 325), 287 (8%)

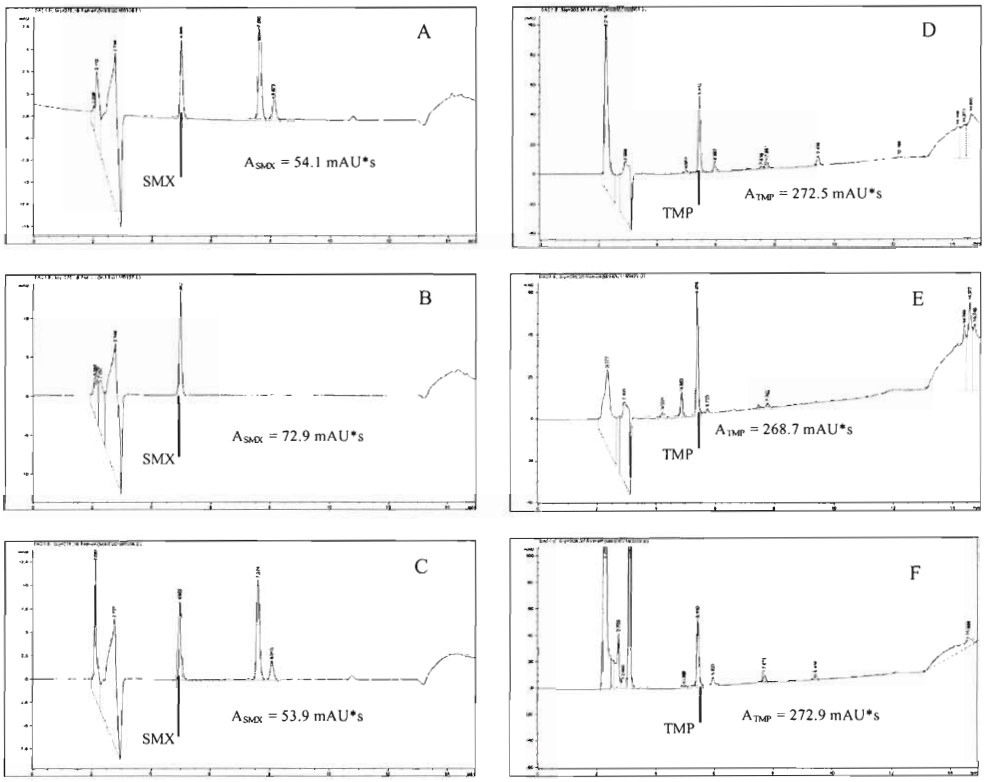


Figure II-1. HPLC-DAD Chromatograms: a) Unquenched SMX reaction solution (2:1 FAC:SMX) at t=10 min., b) SMX reaction solution quenched w/ $Na_2S_2O_3$ at t=10 min., c) SMX reaction solution quenched w/ NH_4Cl, THAM, CH_3COOH at t=10 min., d) Unquenched TMP reaction solution (2:1 FAC:TMP) at t=60 min., e) TMP reaction solution quenched w/ $Na_2S_2O_3$ at t=60 min., f) TMP reaction solution quenched w/ NH_4Cl, THAM, CH_3COOH at t=60 min.

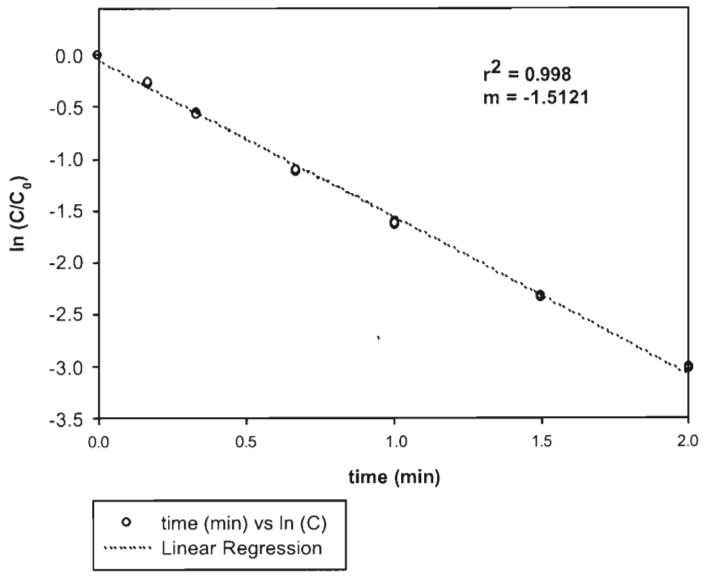
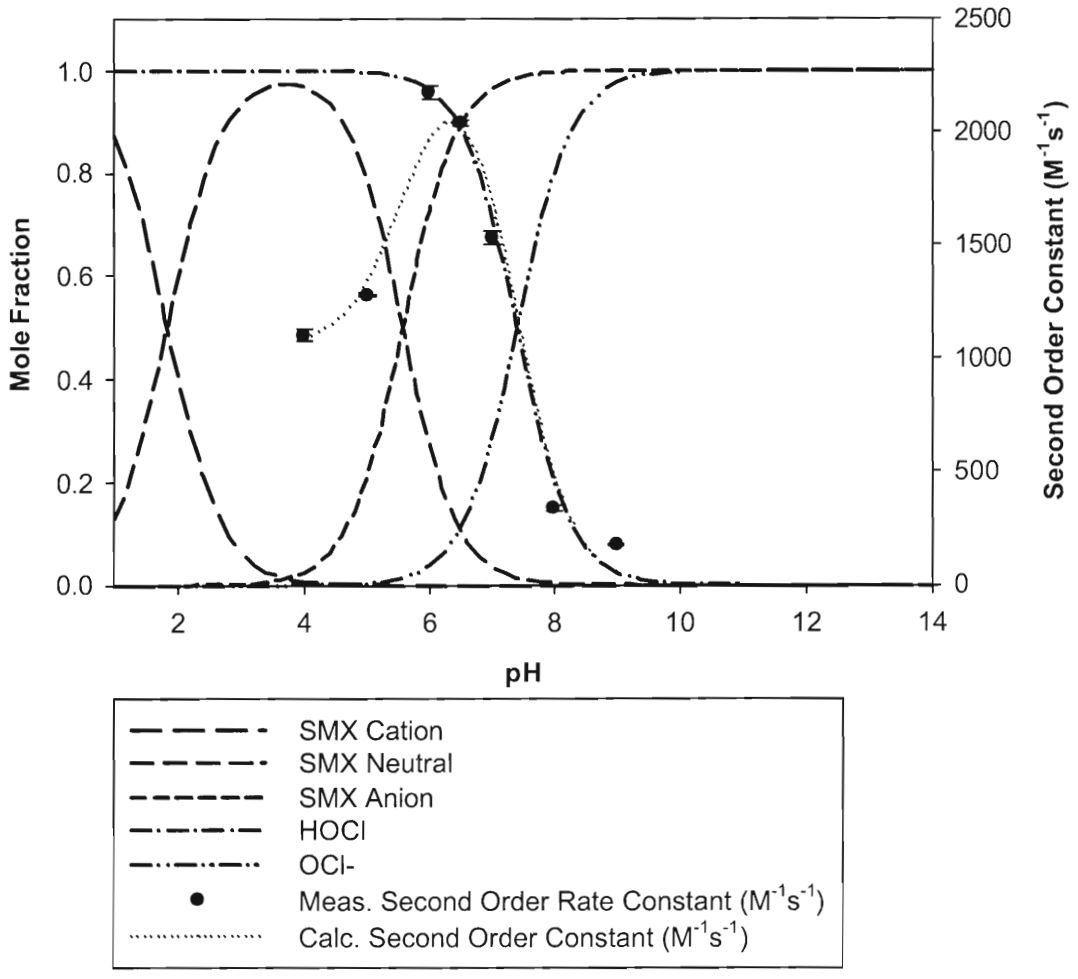


Figure II-2. Determination of pseudo-first order constant for SMX at pH 5 in 0.01 M acetate buffer, C_{SMX} = 0.5 mg/L, C_{FAC} = 1.22 mg/L, 10:1 oxidant:substrate molar ratio



a)

Figure II-3 (continued on next page). pH-dependency of Apparent Second Order Rate Constants for Reaction with FAC: a) SMX and b) TMP

b)

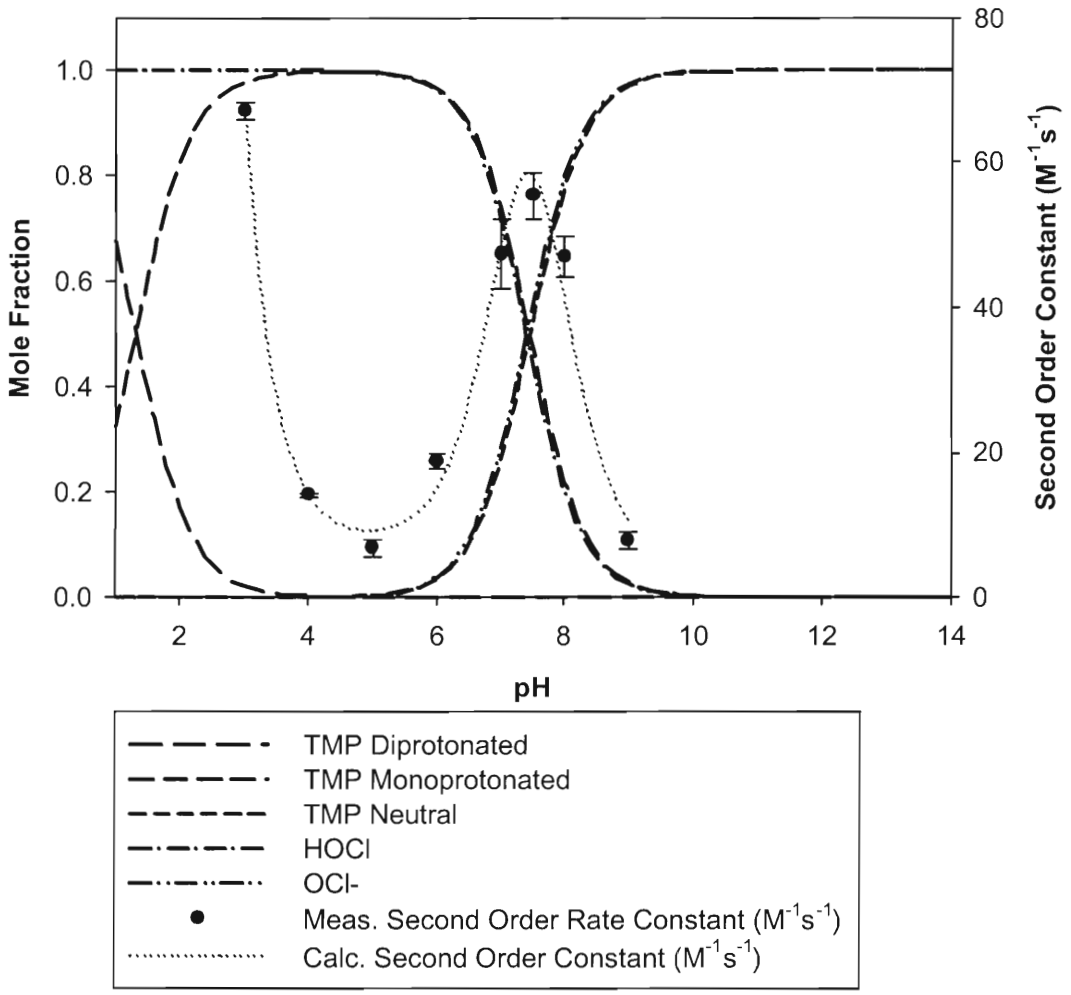


Figure II-3 (continued). pH-dependency of Apparent Second Order Rate Constants for Reaction with FAC: a) SMX and b) TMP

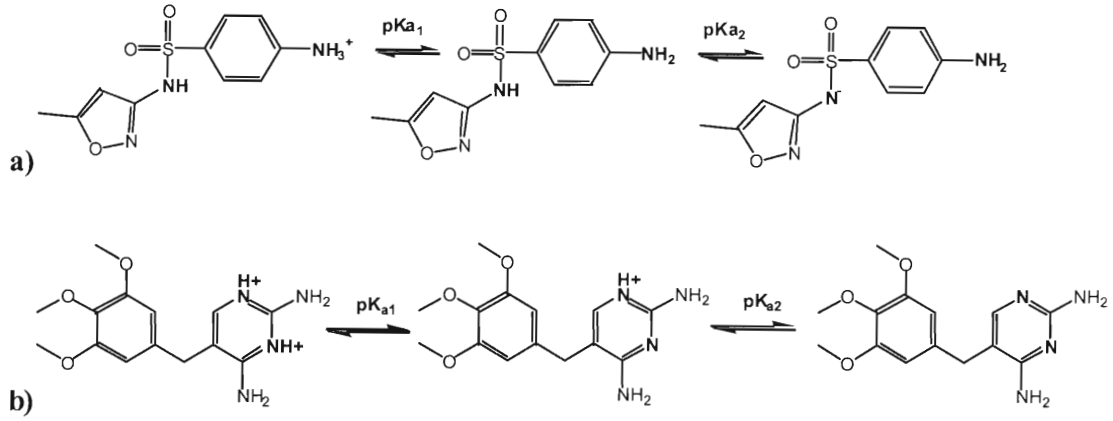


Figure II-4. Speciation Patterns of: a) SMX and b) TMP

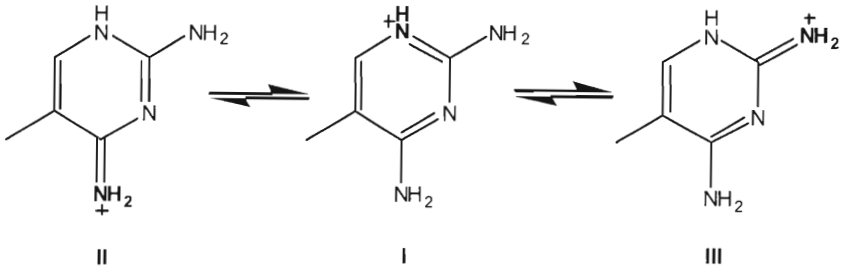
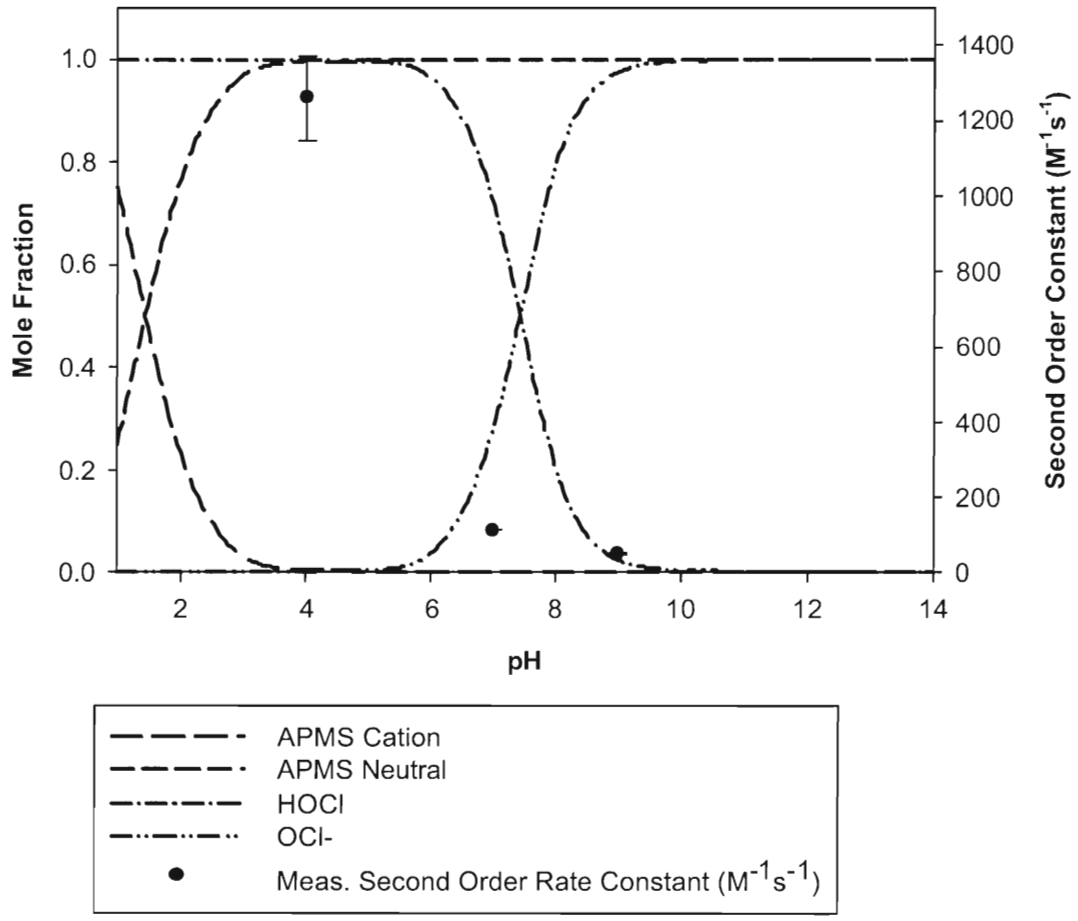


Figure II-5. Resonance stabilization of charge due to protonation of N_1 in 2,4-diaminopyrimidine structure [56]



a)

Figure II-6 (continued on next page). pH-dependency of Apparent Second Order Rate Constants for Reaction with FAC (Substructures and Surrogate Compounds): a) APMS, b) DAMP, c) TMT

b)

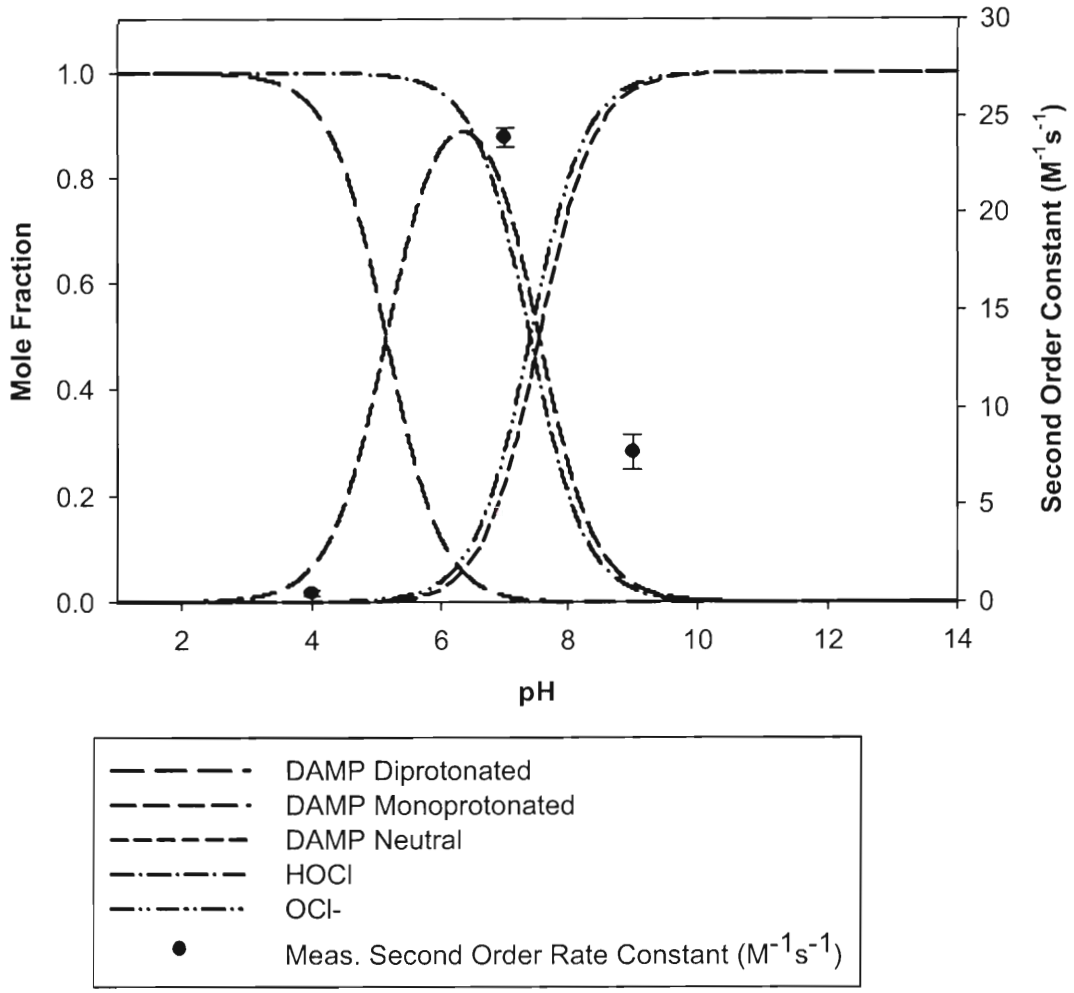


Figure II-6 (continued on next page). pH-dependency of Apparent Second Order Rate Constants for Reaction with FAC (Substructures and Surrogate Compounds): a) APMS, b) DAMP, c) TMT

c)

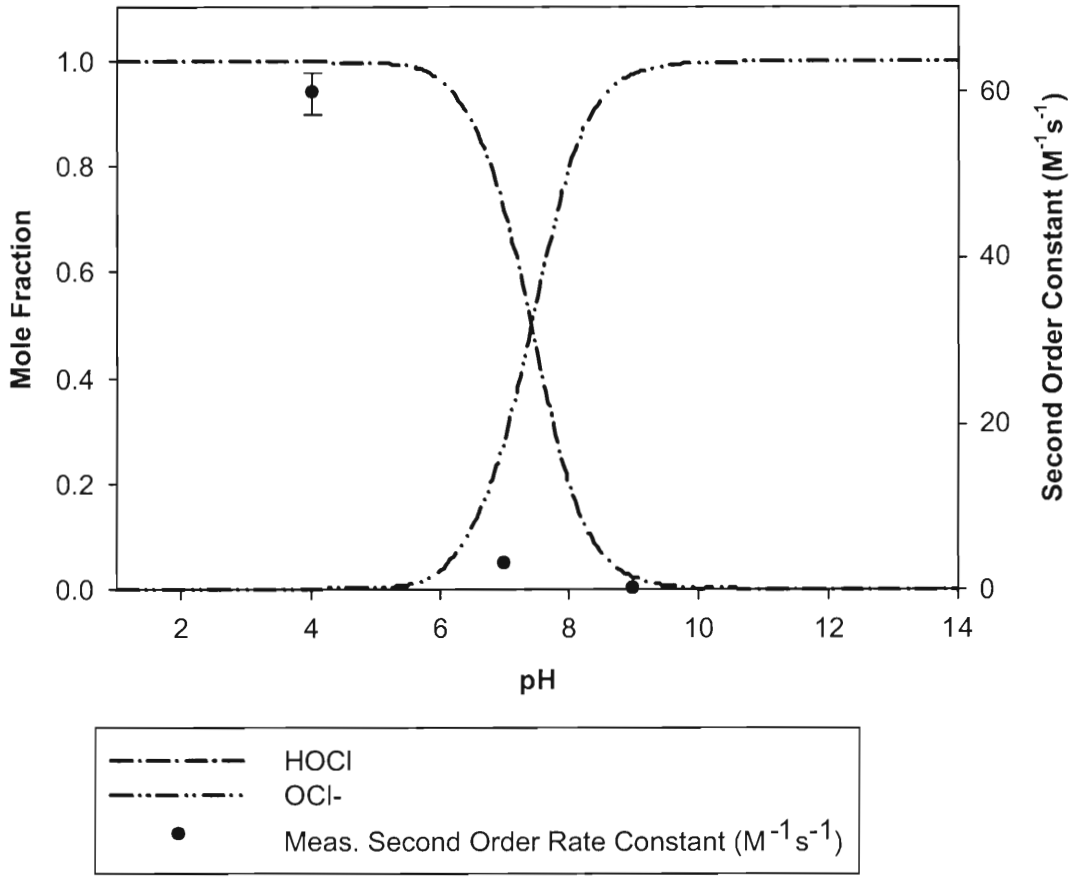


Figure II-6 (continued). pH-dependency of Apparent Second Order Rate Constants for Reaction with FAC (Substructures and Surrogate Compounds): a) APMS, b) DAMP, c) TMT

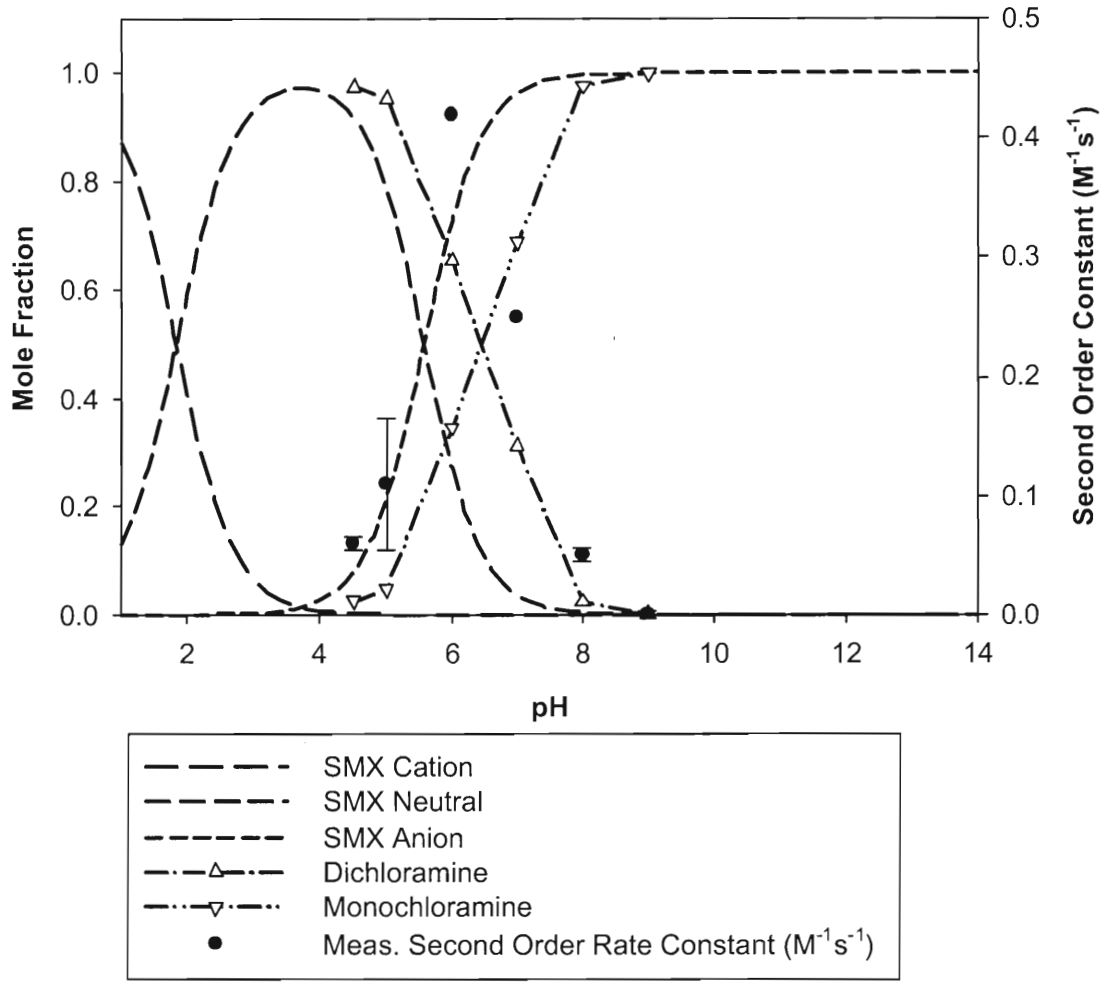
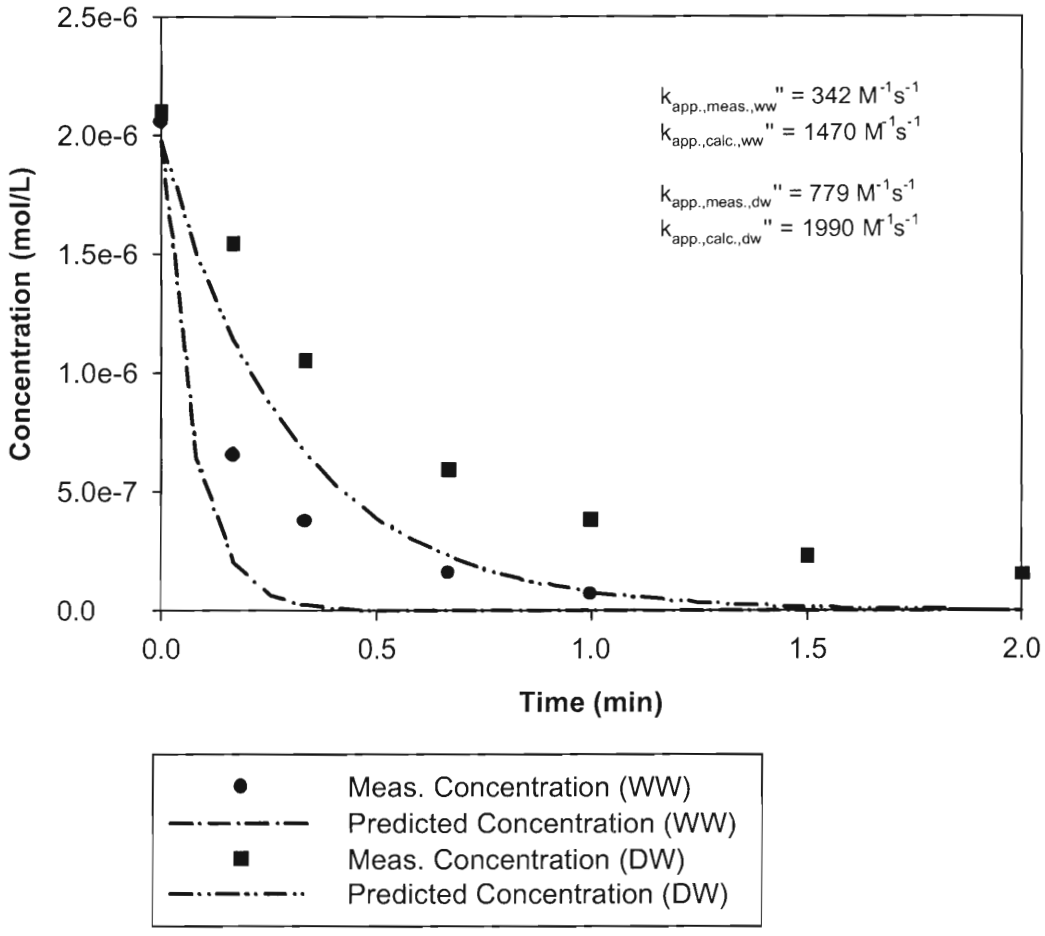


Figure II-7. pH-dependency of Apparent Second Order Rate Constant for Reaction of SMX with CC.



a)

Figure II-8 (continued on next page). Reaction Kinetics in Real Water Systems: a) SMX and b) TMP

b)

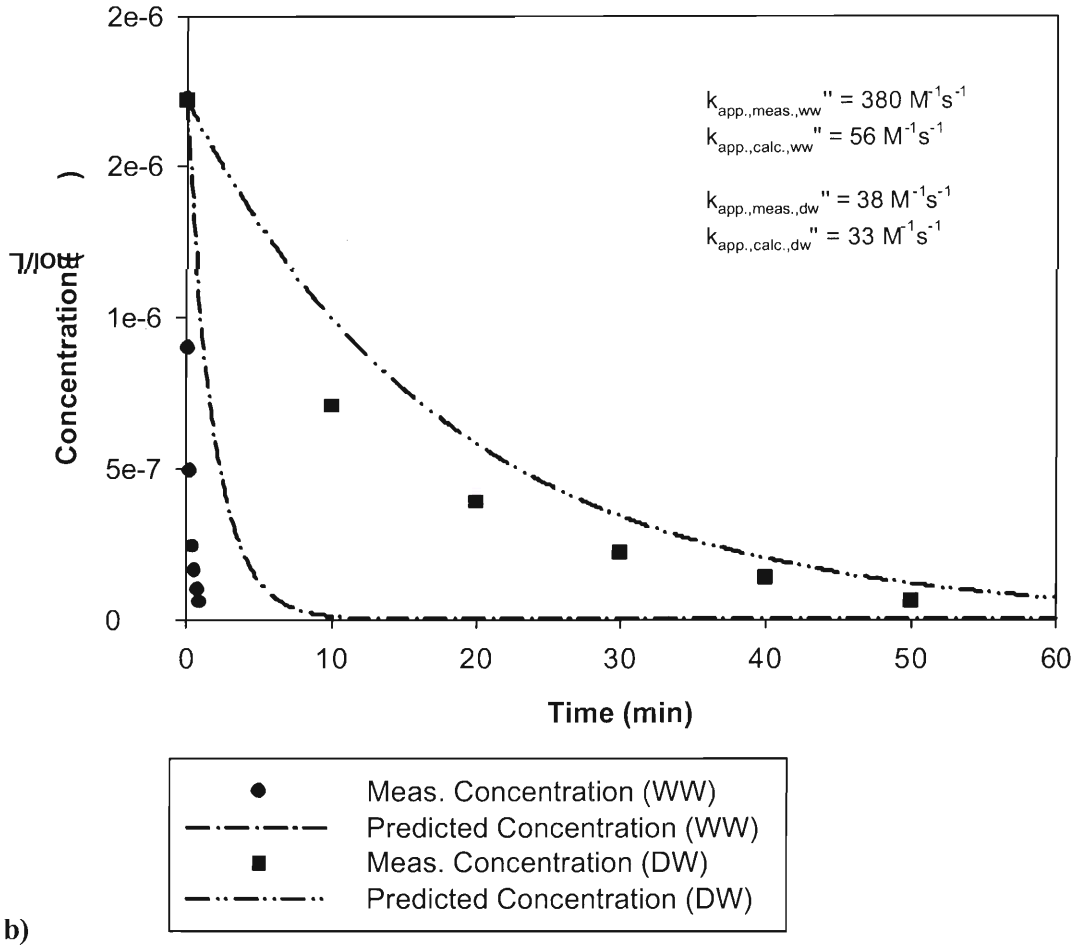


Figure II-8 (continued). Reaction Kinetics in Real Water Systems: a) SMX and b) TMP

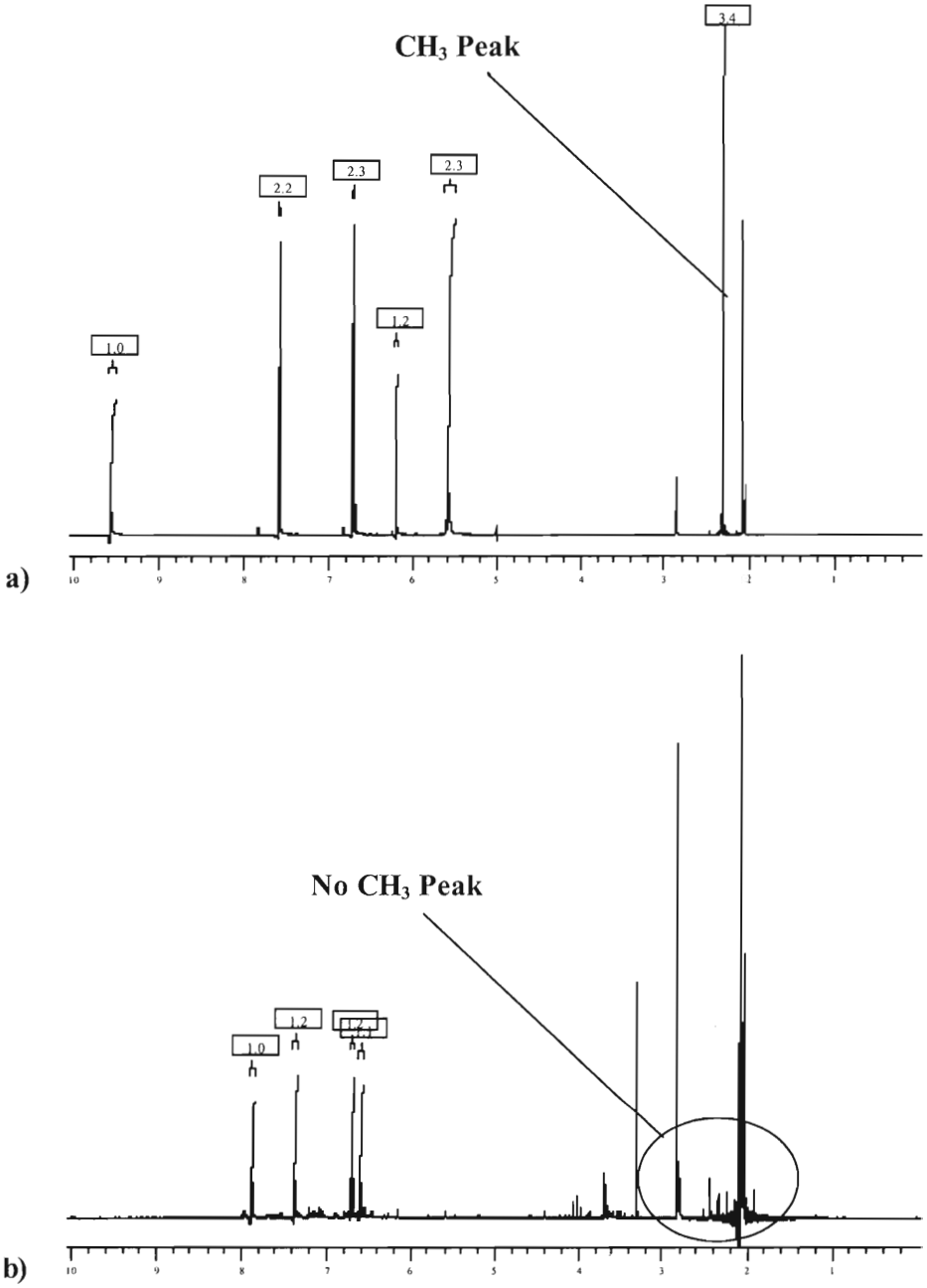


Figure II-9. H-NMR spectra: a) pure SMX, b) SMX Product 1.

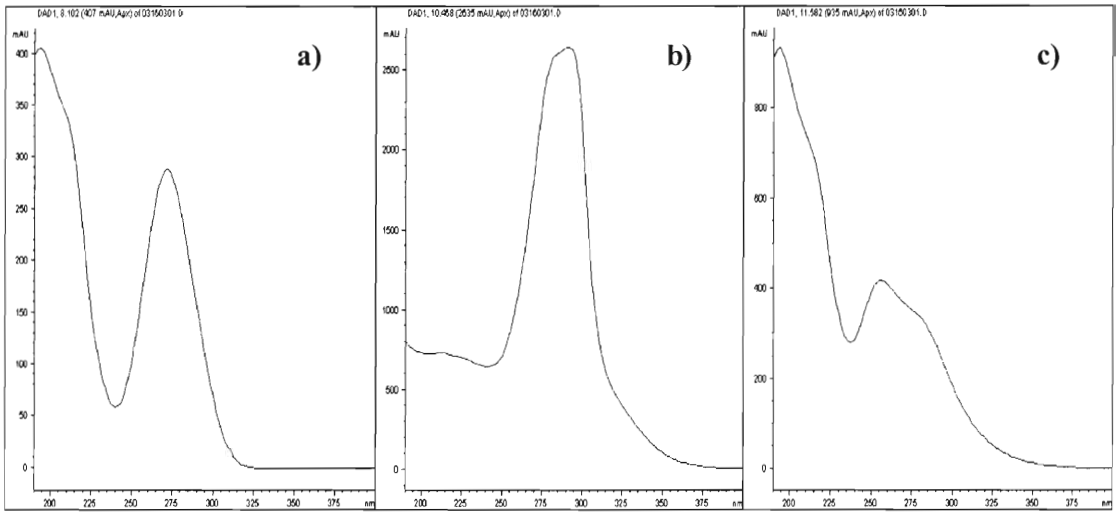


Figure II-10. UV absorption spectra for: a) SMX, b) SMX Product 5, c) SMX Product 7

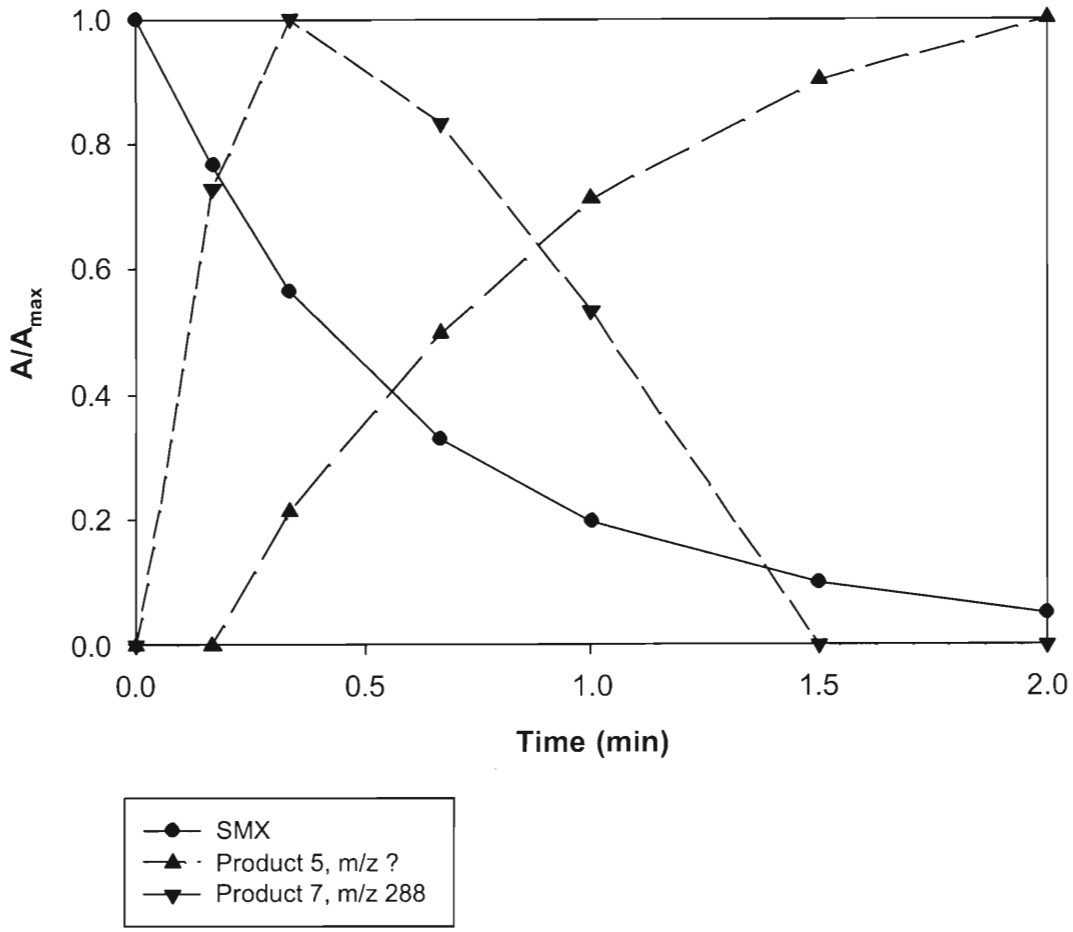


Figure II-11. Major oxidation product evolution for reaction of SMX with FAC (pH 5)

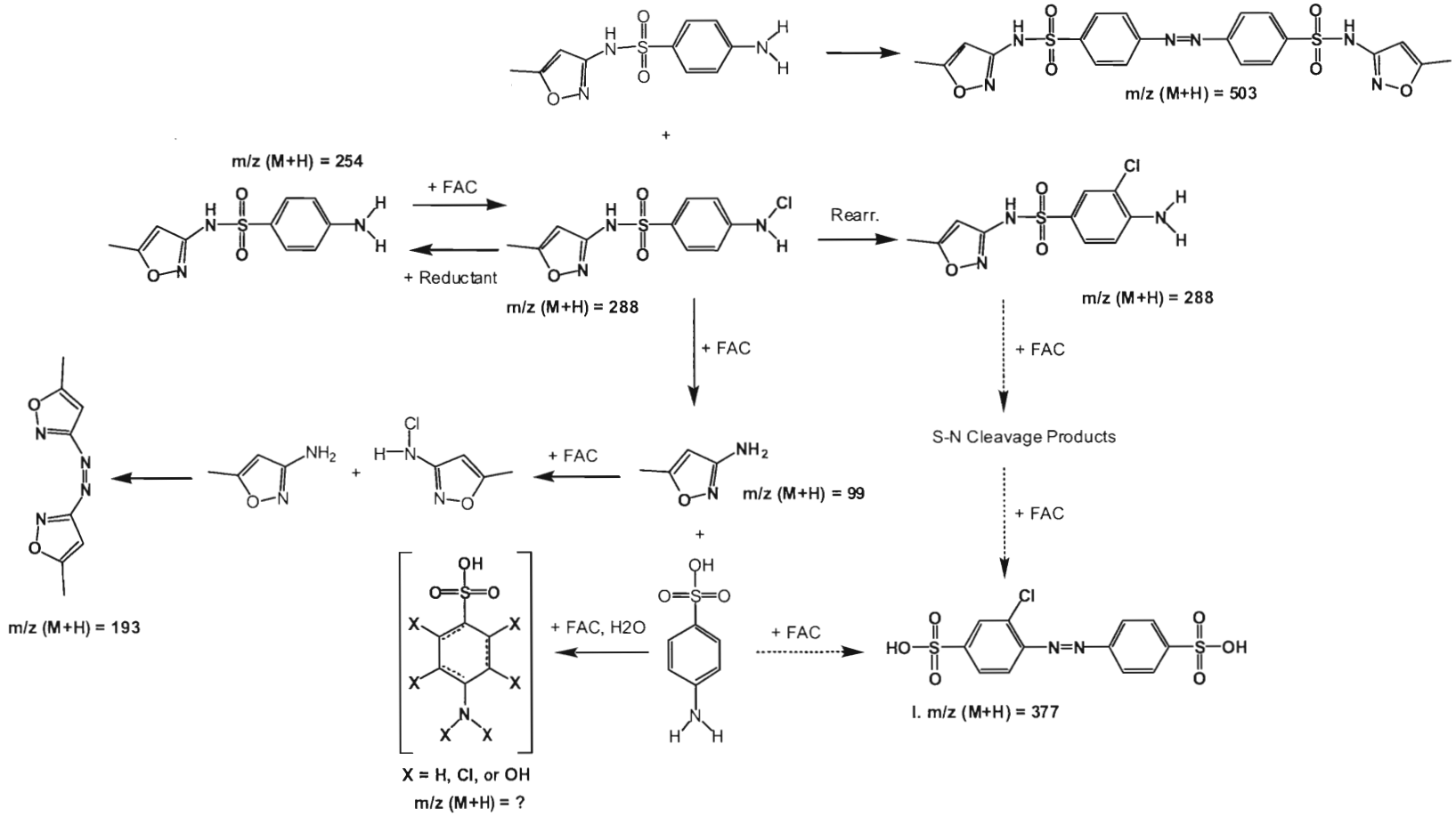


Figure II-12. Proposed degradation pathways for reactions between FAC and SMX

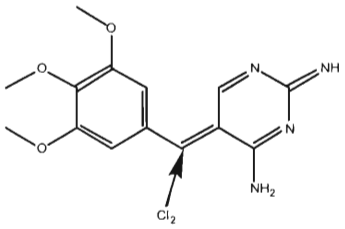
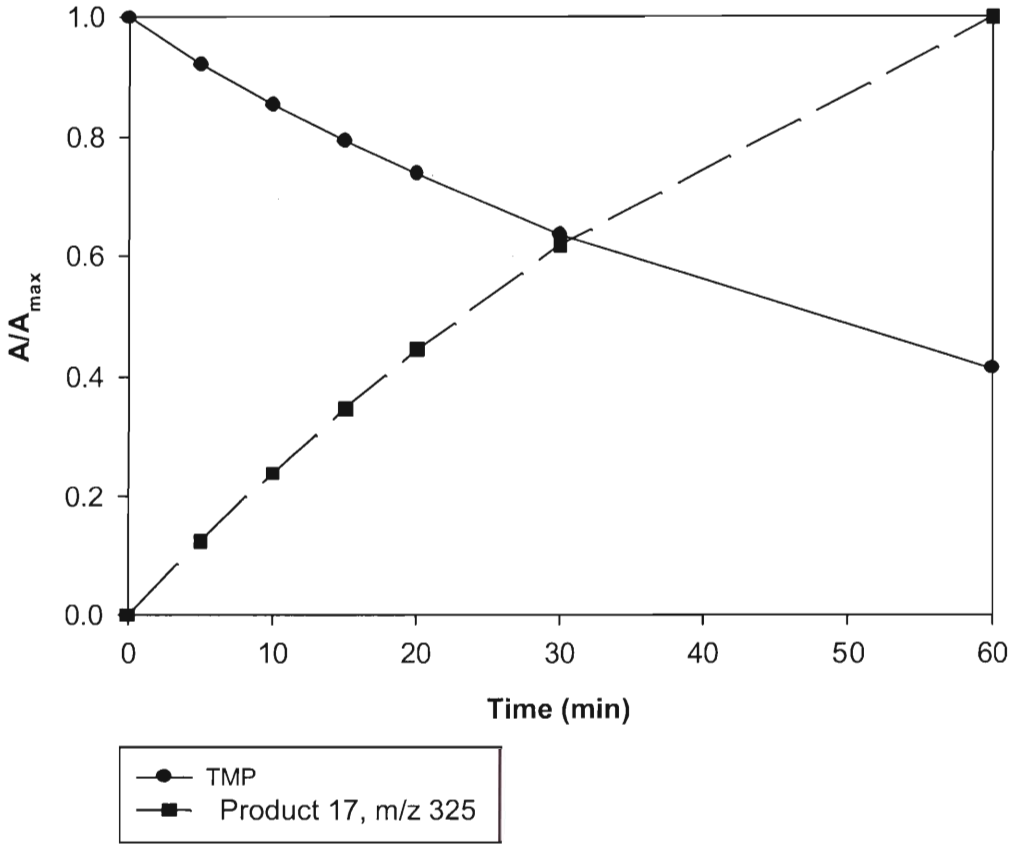
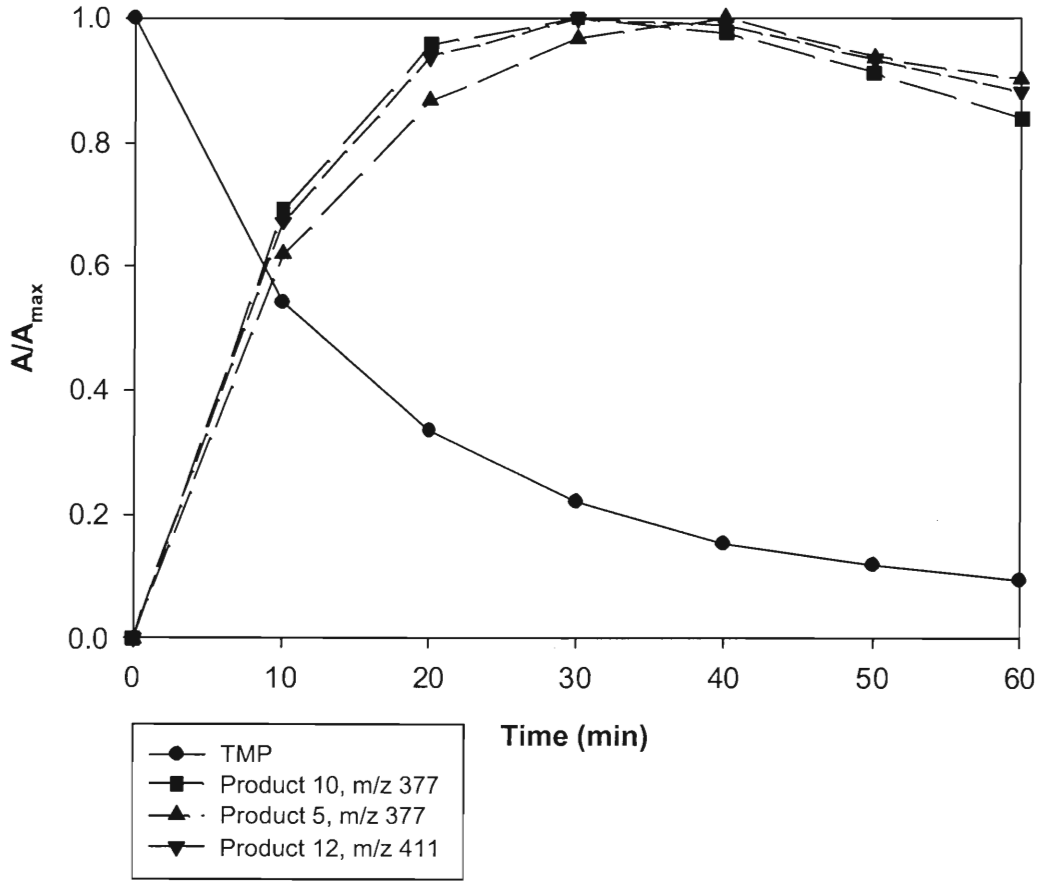


Figure II-13. Proposed TMP iminoquinone methide structure and additional site of chlorine attack.



a)

Figure II-14 (continued on next page). Major oxidation product evolution for reaction of SMX and TMP with FAC: a) TMP/FAC (pH 7) and b) TMP/FAC (pH 4)



b)

Figure II-14 (continued). Major oxidation product evolution for reaction of SMX and TMP with FAC: a) TMP/FAC (pH 7) and b) TMP/FAC (pH 4)

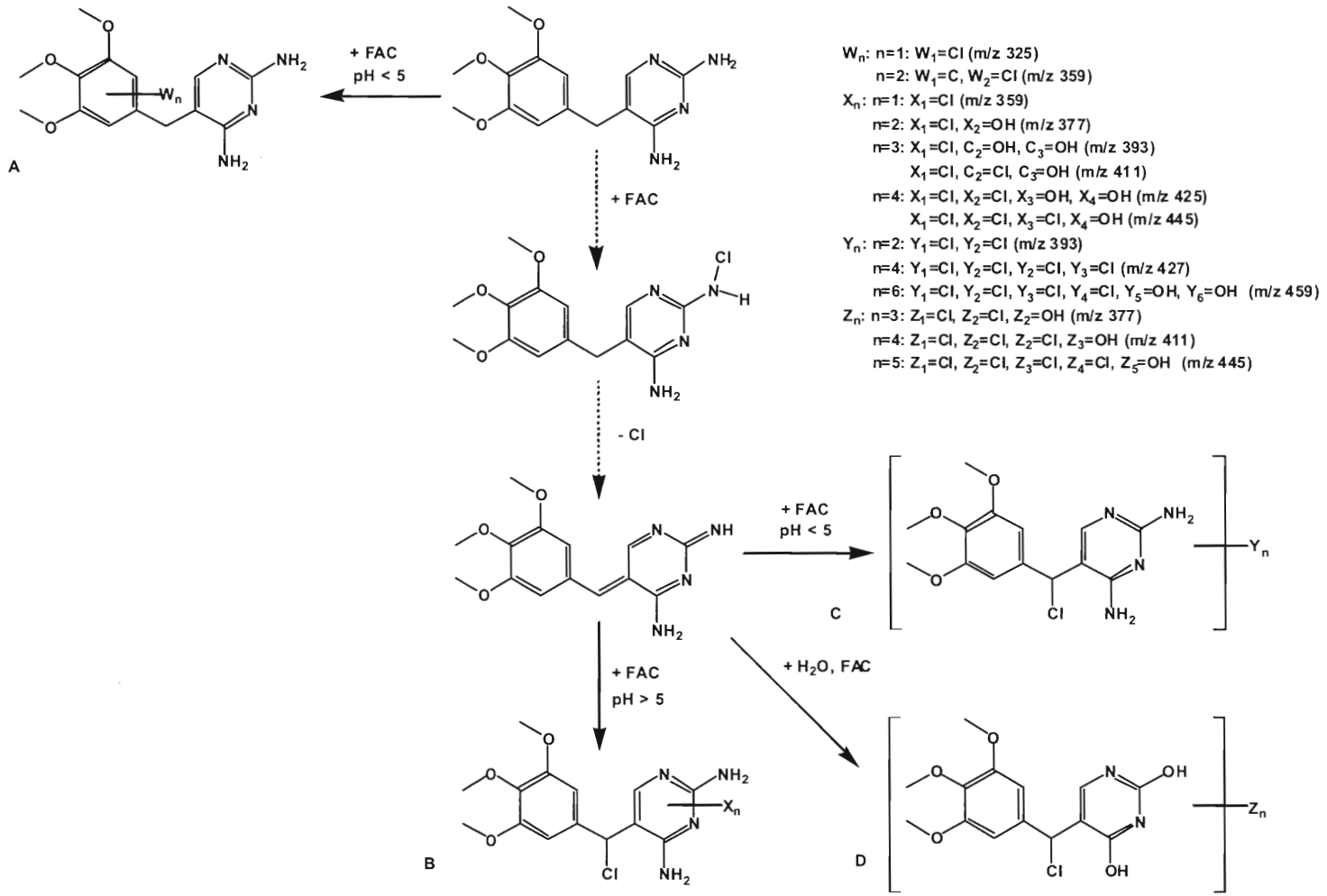


Figure II-15. Proposed degradation pathways for reactions between FAC and TMP

CHAPTER III. CONCLUDING REMARKS

Results. In general, compounds containing amino-nitrogen functional groups are reactive with chlorine oxidants. However, the degree of reactivity for a particular compound – such as CF – can differ significantly from that corresponding to another compound – such as TMP, depending on the presence and/or configuration of various activating and deactivating structural moieties. In the case of CF, a secondary amino group associated with the molecule's piperazine ring imparts extremely high reactivity with FAC. Application of competition kinetics to the qualitative assessment of CF reactivity with FAC indicates a second-order kinetic rate constant well in excess of the resorcinol's measured apparent second order rate constant, which is 5.4 x 10^3 $M^{-1}s^{-1}$ (at pH 7). On the other hand, reactivity of TMP (which contains secondary aromatic heterocyclic nitrogens and primary exocyclic amino groups) with FAC is much slower (characterized by a second order rate constant of approximately 5.5 x 10^1 $M^{-1}s^{-1}$, at pH 7). This is indicative of the relatively wide range of reactivities exhibited by amine-containing nitrogenous substrates, and illustrates the importance of taking into account structure-activity relationships in assessing likely susceptibility of such compounds to oxidative attack by such electrophiles as chlorine. A major objective of this study was to understand the relationships between possession of certain structural features (e.g., primary vs. secondary vs. tertiary amines) and susceptibilities of pertinent antibiotic compounds (or structural surrogates) to FAC. Experimental results indicated a wide range of reactivities for the antibiotic classes studied.

The fluoroquinolone antibiotics CF and LF undergo extremely rapid transformation to N-chlorinated intermediate compounds upon exposure to hypochlorous acid at pH values ranging from mildly acidic to mildly alkaline (pH 4 to 9), which encloses the range of pH likely to be encountered under conditions typifying municipal wastewater and drinking water disinfection processes. The mono-N-chlorinated intermediate of CF (m/z 366) is apparently relatively stable (15 min. < $t_{1/2}$ < 60 min.) , even in the presence of a large excess of oxidant species. Decay of this intermediate in turn appears to represent a rate-limiting step in the overall degradation of CF. The stability of this N-chlorinated intermediate, and its susceptibility to reductive dehalogenation (as noted in Chapter I – Results and Discussion), could indicate that parent CF might be regenerated in conventional wastewater disinfection processes, in which strong reducing agents such as sodium sulfite are used to dechlorinate treated wastewater before ultimate discharge into surface waters. Product characterization for CF reaction mixtures by LC/MS analyses indicates the formation of a number of products, represented primarily by four major degradates corresponding to m/z 263, 297, 306, and 340 (corresponding to full or partial dealkylation of the piperazine ring, and – in two cases – substitution of Cl on the quinolone structure's aromatic ring).

Based on observations recorded in previous studies, and on structural similarities, degradation pathways for LF are expected to be quite similar. The FQs OF and EF undergo much slower initial oxidation by HOCl – presumably due to steric hindrance of HOCl attack on the N_6 piperazine nitrogen due to the presence of tertiary N-alkyl groups – to yield (theoretically) highly transient N-trialkylchloroammonium cation intermediates, which undergo rapid hydrolysis to further yield a series of dealkylated products similar to those produced by

CF. Subsequent degradation of OF and EF is expected to generate oxidation products similar to those identified in CF oxidation experiments.

SMX is oxidized quite rapidly by FAC (10 s < $t_{1/2}$ < 5 minutes) at pH values between 4 and 9, and appears to react most quickly at pH ~ 6.5, which falls within the range of pH values likely to be encountered in water treatment disinfection processes. As noted above for CF, the primary N-chlorinated intermediate of SMX can be reduced relatively easily upon reaction with a strong reducing agent to yield the parent compound, indicating that – at least under conditions of wastewater treatment, for which dechlorination is a relatively common practice after chlorine disinfection – a significant amount of chlorinated SMX could be regenerated prior to final discharge of wastewater effluent into the environment. Experiments conducted in real water systems verify predictions made using data obtained from clean systems. The relatively rapid oxidation of SMX is accompanied by what appears to be a unique radical-chain cleavage of the S-N sulfonamide bond to yield AMI and a modified sulfanilic acid moiety. S-N bond cleavage, combined with N-chlorination of the aniline functional group appears to lead to the formation of relatively stable dimers in addition to lower mass products.

TMP, on the other hand, undergoes relatively slow oxidative transformation compared to SMX (15 min. < $t_{1/2}$ < 60 min.) under the conditions utilized in this study for clean systems, except for pH < 5, for which TMP reactivity with FAC increases significantly. However, owing to undetermined causes, TMP reacts appears to react extremely rapidly in the presence of very large excesses of FAC (i.e., ≫ 10:1 oxidant:substrate molar ratio), as exhibited by its fast degradation in samples obtained from the WRF, and so will likely still be substantially transformed under conditions of both wastewater and drinking water disinfection processes.

The results obtained through completion of the current investigation not only provide information regarding the expected transformation kinetics and pathways of several environmentally significant antibiotic classes (e.g., the fluoroquinolone, sulfonamide, and DHFR inhibitor families), but also provide additional insight into general mechanisms associated with chlorine oxidation of secondary and tertiary amines. In addition, this investigation has highlighted a unique process associated with cleavage of a typically inert sulfonyl amido linkage under relatively mild conditions (neutral pH, moderate temperature, no radical initiator), with respect to FAC oxidation of SMX. Furthermore, the significant differences in degree of structural modification for each antibiotic substrate – as a result of chlorine oxidation – illustrate the importance of understanding not only how rapidly oxidation reactions such as those studied here are likely to proceed in environmental systems, but also what types of compounds such reactions are likely to yield as end-products. In the case of CF and SMX, the parent structures are significantly modified by chlorine oxidation, primarily yielding lower mass degradates. However, the parent TMP structure is relatively recalcitrant in the presence of excess FAC, and rather than degrading to lower mass products, yields a multitude of multiply halogenated and hydroxylated compounds.

Implications. The significance of findings related to fluoroquinolone oxidation – with respect to ecotoxicology or human health risk – is unknown at this point, as a true understanding of the full risks associated with environmental or human exposure to trace amounts of antibiotics or their degradates requires integration of prescription and use indices, environmental fate studies, toxicological data, and exposure models, which was beyond the scope of the current investigation. Previous findings indicate that, while mixtures of photodegradation products (many

of which are similar to chlorination products) corresponding to CF exhibit significantly reduced antimicrobial activity [18, 19], mixtures of OF photodegradation products retain a measurable degree of activity [19]. Thus, incomplete oxidation of FQs (as appears to be the case in chlorination processes), may not completely eliminate the biological effect of these compounds. Subsequent testing to determine the biological significance of FQ degradate mixtures produced by chlorination would certainly be a useful future research pursuit. Although this investigation has contributed to a better understanding of degradation pathways involving oxidation of CF (and other FQs) by FAC, further transformation reactions via such mechanisms as photolysis or metal-oxide facilitated oxidations in the environment could potentially result in more substantial degradation or even mineralization of FQ antimicrobials, as a result of the extended contact times associated with such environmental reactions.

Ecological and human health implications of SMX and TMP oxidation are also unclear at this point, as the confines of this study did not permit a detailed toxicological assessment of reaction products. However, on the basis of observed results, several general inferences can be made. With regard to SMX: As a consequence of the substantial structural modification resulting from reaction of this antibiotic with FAC – the parent molecule's antimicrobial activity is likely to be significantly reduced – if not eliminated – by chlorination. Additional structural characterization of Product 5 is needed in order to fully evaluate the potential significance of SMX degradation products. In contrast to SMX, chlorination of TMP yields primarily stable, multiply-substituted products – at least within the timeframes evaluated in this study – otherwise undergoing relatively minor structural modification. Accordingly, the antimicrobial activity of TMP might not be significantly reduced via chlorination. Additional testing is certainly necessary to determine

the importance of the series of substitution products generated from reactions of SMX and TMP with FAC – both with regard to antibiotic activity and to potential toxicological significance.

Future studies on chemical oxidation of antibiotics or other highly biologically active compounds should take into account not only the degree to which a chemical is oxidized, but also any changes in biological effect resulting from structural modification of the parent molecules. Far too often – as is admittedly the case here, studies of oxidation processes and degradation pathways related to oxidation of particular substrates proceed no further than identification of reaction products, leaving many unanswered questions with regard to the biological or ecological significance of resulting product mixtures.

The use of directed bioassay techniques to assess antimicrobial activity or other modes of toxicity could presumably be applied to mixtures of degradates in order to provide a preliminary assessment of biological effect(s) corresponding to overall mixtures of oxidation products. Information derived from such a preliminary screening method could subsequently be used to determine if further investigation into the properties of relevant degradate mixtures is warranted. In cases for which additional study appears necessary, HPLC fractionation could be utilized in tandem with bioassay techniques to determine which components of a degradate mixture impart significant degrees of biological activity to the bulk mixture. The use of such techniques to provide a means of filtering data related to complex mixtures of reaction products could provide a significant boost to overall throughput in future toxicology screening studies, and would foreseeably impart significantly broader utility to mechanistic chemical oxidation studies such as those conducted as part of the current investigation.

APPENDIX. MATLAB ALPHA MATRIX SOLUTION SCRIPT

```
%Produces a unique solution to a non-homogeneous system of linear
%equations corresponding to the speciation models for pH-dependent mono-
%, di-, and triprotic substrates and oxidants, for a specific condition
%of substrate and oxidant concentrations.

   sub=input('Please enter the name of the substrate of interest:
   ','s');
   ox=input('Please enter the name of the oxidant of interest: ','s');
   subspecies=input('Please provide the number of species constituting
   the speciation character of the substrate: ');
   oxspecies=input('Please provide the number of species constituting
   the speciation character of the oxidant: ');
   data=input('How many apparent second order rate constant values would
   you like to include in this analysis? ');
   a=subspecies*oxspecies;
   Ksubarray=zeros(data,1);
   Koxarray=zeros(data,1);
   nsub=subspecies;
   nox=oxspecies;
   n=1;
   while n<nsub;
      if n==1;

         Ksubarray(n)=10^(-pKsub);
         n=n+1;

         pKsub=input('Please enter the next dissociation constant, K,
         for the substrate: ');
         Ksubarray(n)=10^(-pKsub);
         n=n+1;
      end
   end
   n=1;
   while n<nox;
      if n==1;

         Koxarray(n)=10^(-pKox);
         n=n+1;

         pKox=input('Please enter the next dissociation constant, K, for
         the oxidant: ');
         Koxarray(n)=10^(-pKox);
         n=n+1;
      end
   end
   n=1;
```

```
    while n<=data;
       if n==1;

          pH=input('To what pH value does this correspond? ');
          Harray(n)=10^(-pH);
          n=n+1;

          Kapp(n)=input('Please enter another apparent second order order
          constant, Kapp (1/(M*s)), for the substrate: ');
          pH=input('To what pH value does this correspond? ');
          Harray(n)=10^(-pH);
          n=n+1;
       end
    end
    n=1;
    while n<=data;

alphasub0(n)=Harray(n)^3/(Harray(n)^3+Ksubarray(1)*Harray(n)^2+Ksubarray
(1)*Ksubarray(2)*Harray(n)+Ksubarray(1)*Ksubarray(2)*Ksubarray(3));

alphasub1(n)=(Ksubarray(1)*Harray(n)^2)/(Harray(n)^3+Ksubarray(1)*Harray
(n)^2+Ksubarray(1)*Ksubarray(2)*Harray(n)+Ksubarray(1)*Ksubarray(2)*Ksub
array(3));

alphasub2(n)=(Ksubarray(1)*Ksubarray(2)*Harray(n))/(Harray(n)^3+Ksubarra
y(1)*Harray(n)^2+Ksubarray(1)*Ksubarray(2)*Harray(n)+Ksubarray(1)*Ksubar
ray(2)*Ksubarray(3));

alphasub3(n)=(Ksubarray(1)*Ksubarray(2)*Ksubarray(3))/(Harray(n)^3+Ksuba
rray(1)*Harray(n)^2+Ksubarray(1)*Ksubarray(2)*Harray(n)+Ksubarray(1)*Ksu
barray(2)*Ksubarray(3));
    n=n+1;
    end
    n=1;
    while n<=data;

alphaox0(n)=Harray(n)^3/(Harray(n)^3+Koxarray(1)*Harray(n)^2+Koxarray(1)
*Koxarray(2)*Harray(n)+Koxarray(1)*Koxarray(2)*Koxarray(3));

alphaox1(n)=(Koxarray(1)*Harray(n)^2)/(Harray(n)^3+Koxarray(1)*Harray(n)
^2+Koxarray(1)*Koxarray(2)*Harray(n)+Koxarray(1)*Koxarray(2)*Koxarray(3)
);

alphaox2(n)=(Koxarray(1)*Koxarray(2)*Harray(n))/(Harray(n)^3+Koxarray(1)
*Harray(n)^2+Koxarray(1)*Koxarray(2)*Harray(n)+Koxarray(1)*Koxarray(2)*K
oxarray(3));

alphaox3(n)=(Koxarray(1)*Koxarray(2)*Koxarray(3))/(Harray(n)^3+Koxarray(
1)*Harray(n)^2+Koxarray(1)*Koxarray(2)*Harray(n)+Koxarray(1)*Koxarray(2)
*Koxarray(3));
    n=n+1;
    end
alphasub=[alphasub0' alphasub1' alphasub2' alphasub3'];
alphaox=[alphaox0' alphaox1' alphaox2' alphaox3'];
```

```
m=1;
n=1;
nn=1;
o=1;
while m<=data
while o<=oxspecies
while nn<=subspecies
   alpha(m,n)=alphasub(m,nn)*alphaox(m,o);
   n=n+1;
   nn=nn+1;
end
nn=1;
o=o+1;
end
m=m+1;
n=1;
nn=1;
o=1;
end
n=1;
pcnum=input('Please provide the number of principal variables
constituting the system reaction character: ');
   while n<=pcnum;
      if n==1;

         PCarray(n)=PC;
         n=n+1;

         PC=input('Please enter the number corresponding to the next
         principal variable (or sub-reaction): ');
         PCarray(n)=PC;
         n=n+1;
      end;
   end;
n=1;
   while n<=pcnum
      alphared(:,n)=alpha(:,PCarray(n));
      n=n+1;
   end;
solution=alphared\Kapp';
```

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